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BIOSYNTHESIS OF IRON(Fe) NANOPARTICLES AND ITS INHIBITORY EFFECT ON PSEUDOMONAS AERUGINOSA BIOFILM

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ABSTRACT Biofilms are the complex bacterial communities which resist the mode of action of antibiotics and the immune system of the human also. Microorganisms unanimously attach to the surfaces and produce extracellular polysaccharides, resulting in the development of a biofilm. Biofilms create a severe trouble for public health because of the increased rate of resistance of biofilm associated organisms. The chronic infection related to biofilm by *Pseudomonas aeruginosa* are always hard to be cured because of their inherent resistance to both antimicrobial agents and host defense. The present study is devoted to the possibility of metal nanoparticles synthesis using plant extracts and its inhibitory effect on *Pseudomonas aeruginosa*. Here Iron nanoparticles were synthesized using *Annona squamosa* which was characterized by using UV-Visible Spectroscopy analysis and Fourier Transform Infrared Spectroscopy (FTIR). To evaluate the formation of *Pseudomonas aeruginosa* biofilm and the activity of Iron nanoparticles against the biofilm and they showed good anti-biofilm activity.

KEYWORDS : *Annona squamosa*, anti-biofilm, Iron, Pseudomonas aeruginosa and Fourier Transform Spectroscopy

Introduction

In both inside and outside role of the medicine, the administration of antibiotic in widespread manner which was highly plays a vital role into the multidrug resistant bacteria. Antibiotic resistance is a kind of drug resistance anywhere a microorganism is competent to survive to the exposure of an antibiotic (Gossens *et.al,* 2005; Motta *et.al*, 2003; Mohammed Azam Ansari *et.al,* 2013). Resistance in human pathogens is a big challenge in the field like pharmaceutical and biomedicine. Antibiotic resistance profiles lead to fear about the emergence and re-emergence of multidrug resistant (MDR) pathogens and parasites (Tenover, 2006 and Ashajyothi *et.al*, 2015). In recent years, the number of infections associated with antibiotic resistant bacteria has increased. Many of these infections are caused by microorganisms growing in biofilms. Both gram positive and gram negative bacteria can form biofilms on indwelling medical devices such as catheters, mechanical heart valves and prosthetic joints. The most common biofilm forming bacteria associated with human diseases *Enterococcus faecalis, Staphylococcus aureus, Klebsiella pneumonia, Staphylococcus epidermis, Staphylococcus viridians, Proteus mirabilis, Pseudomonas aeruginosa* (Donlan, 2001 and Katarzyna *et.al*, 2013). Biofilm related diseases persistent infections characterized by slow development, an ability to resist post immune defenses and a transient response to anti microbial therapy (Parsek & Singh, 2003 and Katarzyna *et.al*, 2013).

The therapeutic efficacy of antibiotics correlates closely with their bactericidal or bacteriostatic effects. Bacteria can, however, adapt to the selective pressure from antibiotics via genetic alteration leading to development of antibiotic resistance. The efficiency of many traditional antibiotic treatments is currently decreasing, while the occurrence of multiple resistance pathogenic bacteria is increasing. *Pseudomonas aeruginosa* is one of the most common gram negative bacteria found in association with nosocomial infections and in immuno-compromised patients (Hoiby *et.al*, 2000 and Wu *et.al*, 2004). *Pseudomonas aeruginosa* is a bacterium well documented to form biofilms on moist surfaces, medical instruments and in chronic respiratory infections, particularly in cystic fibrosis patients (Djeribi *et.al*, 2012, Bryers, 2008, Bjarnsholt *et.al*, 2009 and Greg Tram *et.al*, 2013). Biofilms will form on almost any material where nutrients available, but it happens more likely, if the attachment surface is rough, scratched, cracked, or corroded. Physical conditions, such as hydrophobicity, surface electrostatic charge, and fluid flow rate also affect the attachment. Several studies have shown that microorganisms attach more rapidly to hydrophobic, non-polar surfaces such as Teflon and other plastics than to hydrophilic surfaces like stainless steel so some kind of hydrophobic interaction apparently occurs, which enable the cells to overcome the repulsive forces (Donlan, 2002).

Nanotechnology is another approach for the development of novel non-traditional antimicrobial agents. This new paradigm designs new antimicrobial drugs- "nano-antibiotics". Nanomaterials, which either show antimicrobial activity by themselves or enhance the effectiveness and safety of antibiotic administration, are called "nano-antibiotics" (Hajipour *et.al*, 2012). They possess many advantages over other antimicrobial agents including increasing effectiveness against drug-resistant species, lack of adverse effects, and overcoming resistance development interfering with a multiple of biological pathways (Huh *et.al*, 2011). Nanotechnology is an emerging field to discover, describe and manipulate the unique properties of matter, especially metals at the nano-scale in order to develop new capabilities with applications across all fields of science, engineering and medicine (Nair *et.al*, 2001 & Sharma *et.al*, 2009).

Metal nanoparticles are currently used in different fields for their unique properties (Mohanpuria *et.al*, 2008, Kiruba Daniel *et.al*, 2013 and Bhuvaneswari *et.al*, 2015). The biological synthesis of nanoparticle is a challenging concept which is very well known as green synthesis. Biosynthesis of nanoparticles could be an alternative to traditional chemical methods for the production of metallic nanomaterials in a clean, nontoxic and ecologically sound manner. Green synthesis of nanoparticle is cost effective, easily available, eco friendly, non-toxic, large scale production can be done easily and acts as reducing and capping agent when compared to the chemical method which is a very costly as well as emits hazardous by-products which can have some deleterious effects on the environment. Nanoparticles synthesis using plants provides more biocompatible nanoparticles than chemical synthesis, while chemical synthesis may precedence to the existence of some toxic chemical species on the surface of nanoparticles that may have defective effects in biomedical applications (Ahmad *et al.,* 2011 and Sravanthi *et.al*, 2016). In recent times, the utilization of different plant materials for the biosynthesis of nanoparticles is contemplated a green technology because it does not evolve any harmful chemicals. Among them, Iron nanoparticles (Fe NPs) are gaining importance for their use in environmental remediation technologies. In this current study, iron oxide nanoparticles (Fe_3O_4-NanoParticles) were synthesized by means of a rapid, single step and absolutely green biosynthetic method by reduction of ferric chloride solution (Sravanthi *et.al*, 2016). Hence, the aim of present study is to synthesize Iron nanoparticles by using *Annona squamosa* and study the anti biofilm action against *Pseudomonas aeruginosa.*

Plant Description

Kingdom: Plantae
Order : Magnolids
Family : Magnoliales
Genus : Annona
Species : *Annona squamosa*

Materials and Methods

Reagents and Chemicals

0.001M Ferric chloride was obtained from Sigma Aldrich. Freshly

prepared Distilled water was used throughout the experiment.

Preparation of Plant extracts

20grams of fresh *Annona squamosa* leaves were cut and thoroughly washed with distilled water. The weighed leaves were allowed to mix with the 100ml of distilled water and boiled for 60mins as reported earlier (*Daniel et.al*, 2012). The broth extract was filtered using Whatman No.1 filter paper and kept at 4°c for future use.

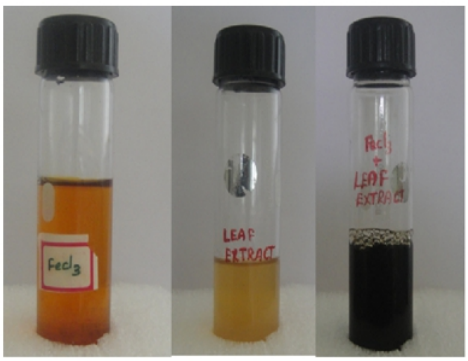

Figure 1: shows the synthesis of nanoparticles ($FeCl_3$ solution, Leaf extract and mixture of leaf extract with $FeCl_3$)

Synthesis of Fe Nanoparticles

Iron nanoparticles were synthesized by adding 2ml of $FeCl_3$ solution with 20ml of *Annona squamosa* leaf extracts (Bhuvaneswari *et.al*, 2015). A change in color from faint yellow to brownish yellow and finally dark after certain period of time which indicates the formation of iron nanoparticles (shows Fig-1). The Iron nanoparticles solution was allowed to purify by centrifugation at 12,000 rpm for 15min followed by re-dispersion of the pellet in deionized water (Pramila and Meenakshisundaram, 2016). Then the Iron nanoparticles were dried in oven at 80°c and stored in air tight container for further analysis.

UV-Vis spectroscopy analysis

The reduction of Iron nanoparticles was measured by UV-Vis spectrum. 0.3ml of sampling aliquots of Iron nanoparticles solution was diluted with 3ml of distilled water (Pattanayak *et.al*, 2013). UV-Vis spectral analysis was done by using at the range of 200-600nm, the absorption peak regions was observed, due to the excitation of the surface Plasmon vibrations in the Fe nanoparticles solution, which are the characteristics of metallic Iron nanoparticle and finally it was recorded.

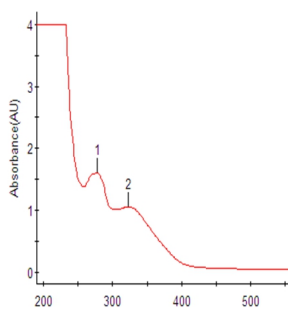

Figure 2 : UV-Vis spectra of Control (Leaf extract)

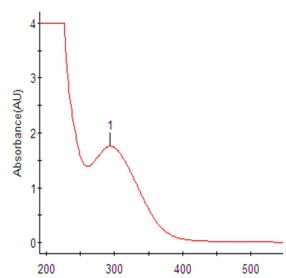

Figure 3: UV-Vis spectra of Test (FeCl3 + Leaf extract)

FTIR analysis

The molecular functional groups as Phyto-constituents present in the Iron nanoparticles were determined by Fourier Transform Infrared Studies (FT-IR). FT-IR analysis was carried out by spectrum RX-1 instrument in diffuse reflectance mode operated at a resolution of 4cm of wavelength of about 4000-400 cm using KBr pellets (Senthamilselvi et.al, 2013).

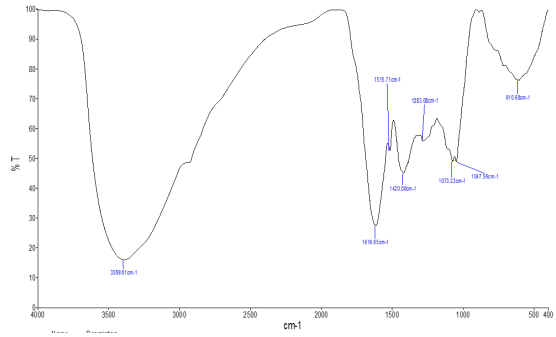

Figure 4: FTIR spectra of the control- Leaf extract

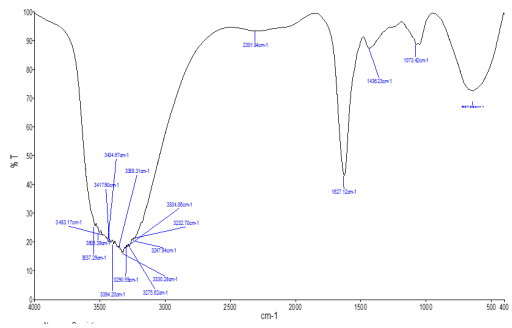

Figure 5: FTIR spectra of the Fe nanoparticle (Test- leaf extract with $FeCl_3$ solution)

Tube method

A qualitative assessment of biofilm formation was determined as previously described by (Christensen *et.al*, 1985) Nutrient broth (10ml) was inoculated with loopful of microorganisms (*Pseudomonas aeruginosa*) from overnight culture plates and incubated for 24 hours at 37°c. The tubes were decanted and washed with PBS (pH 7.3) and dried. Then, the dried tubes were stained with crystal violet (0.1%). Excess stain was removed and tubes were washed with deionized water. Tubes were than dried in inverted position and observed for biofilm formation. Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Tubes were examined and the amount of biofilm formation was performed in triplicate and repeated three times (Mathur *et.al*, 2006).

Figure 6: Tube method (control- Overnight culture, then stain with 1% crystal violet and Test- Overnight culture with Fe nanoparticles, then stain with 1% crystal violet)

Micro-titre Dish assay

The overnight culture of Pseudomonas aeruginosa was added with the various concentration Fe nanoparticles from Annona squamosa to the 96-well plates after 24 hours of incubation which containing formed biofilm were then washed with distilled water three times to remove the excess culture. A 1% crystal violet solution (Sigma) was then used to stain the remaining biofilm and allowed to penetrate for 15 minutes.

The wells were then washed a further 3 times with distilled water (Greg et.al, 2013). Then the rate of biofilm formation was recorded.

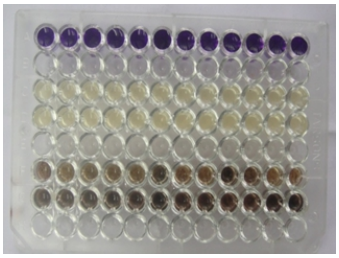

Figure 7: shows the micro titre plate method- The 1st row of the disk contain crystal violet alone,

3r[d] & 4[th] row contains Overnight culture alone, 6[th] row contain overnight culture and Fe nanoparticles in the ratio of (1:1) and 7th row contain overnight culture and Fe nanoparticles in the ratio of (1:2)

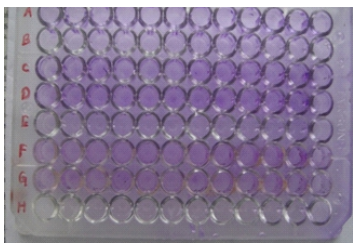

Figure 8: shows after incubation of micro titre plate method for the detection biofilm formation and anti biofilm activity using Fe nanoparticles

(Row A- crystal violet alone, C&D- Overnight culture of Pseudomonas aeruginosa alone,
F- 1:1 ratio of culture with Fe nanoparticles and G- 1:2 ratio of culture with Fe nanoparticles)

Anti microbial Activity

The anti microbial activity was done by using *Pseudomonas aeruginosa* culture against Fe nanoparticles. The overnight culture was sub cultured on Nutrient broth at 37°c and allowed to incubate overnight. The culture was spread uniformly onto the individual Nutrient agar plates using sterile cotton swab (Ramalingam *et.al*, 2014 and Kalishwaralal *et.al*, 2010). Well of approximately 5 mm diameter was made on Nutrient agar plates using gel puncture. 20µl of synthesized Fe nanoparticles was inoculated into the well and then the plates were incubated at 37°c for 24 hours and the formation of the zone of inhibition was monitored.

Figure 9: Antimicrobial activity (Control-Without adding Fe Nanoparticles)

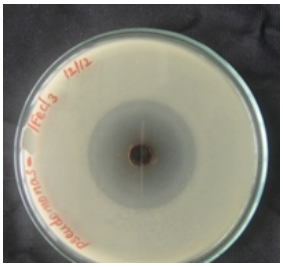

Figure 10: Antimicrobial activity- Clear zone using Fe nanoparticles against Pseudomonas aeruginosa

Results and Discussion

UV–Vis absorption spectroscopy is one of the main tools to analyze the formation of metal nanoparticles in aqueous solutions (Wiley et al. 2006). The biologically synthesized iron nanoparticles were analyzed using ultra violet-visible spectroscopy (UV-Vis) in 300 nm to 700 nm wave length range. The spectra clearly show maximum absorption peaks which indicate the formation of increased number of iron nanoparticles in the solution (Chandran *et.al*, 2016). Harshiny *et.al*, 2015 said that the formation of Fe Nanoparticles was monitored by UV-Vis spectrophotometer wavelength ranges from 200-800 nm. An absorption peak at 214 nm and 260 nm corresponds to Nanoparticles and the presence of peptide bonds respectively. In this present study the UV–Vis spectrum of extract and $FeCl_3$ mediated Nanoparticles confirmed the formation of Fe by the absorbance peak at 271 nm and the control shows a maximum absorbance peak at 250 and 365nm as shown in Fig 2&3. The broad absorption spectrum of Fe Nanoparticles may be due to its size and aggregation of nanoparticles.

The interaction of aqueous leaf extract on the surface of Nanoparticles was confirmed by FTIR characterization. FTIR spectra of biosynthesized Iron oxide nanoparticles were recorded to identify the capping and efficient stabilization of metal nanoparticles by the functional groups of biomolecules present in the leaf extract which gives various peaks related to O-H bond at the range from 3247.9-3537.2, 2301.3 denotes C-C bond, 1627.1 sharp peak denote N-H stretching and 1436.2 is associated to sp^3 C-H bend. The peak was highly differ from the control which highly associated with the water molecules with C=O and N-H stretching. Fig 4&5 shows the FT-IR spectrum of prepared iron nanoparticles. FTIR is an analytical instrument plays a major role in determining the functional groups of many organic and inorganic compounds. The wavelength of light absorbed is characteristic of the chemical bond as can be seen in the annotated spectrum. By interpreting the infrared absorption spectrum, the chemical bonds in a molecule can be determined [Ashokkumar *et.al*, 2014]. The tube adherence test depends on the visual assessment of the degree of adherence of *Pseudomonas aeruginosa* to the sides of borosilicate test tubes (Fig-6). In micro titer plate method the biofilm formation was highly inhibited by the activity of Fe nanoparticles which was confirmed by the intensity of colour present in the 96 well micro titer disks (Fig 7&8). The antimicrobial study of green synthesized Fe nanoparticles using disk diffusion method as shown in Fig-9 shows the zone of inhibition (ZOI) for Fe nanoparticles, it creates 3cm of zone. In both tube method and micro titre plate method the culture with Fe nanoparticle incorporation will give maximum anti biofilm activity. This result was effective when the concentration of Fe nanoparticles was observed to be increased with increase in the zone of inhibition.

Conclusion

We have successfully synthesized Fe nanoparticles using the *Annona squamosa* leaf extract. The biological approach of synthesis of Iron nanoparticles using *Annona squamosa* appears to be ecofriendly and cost effective alternative to conventional chemical and physical methods and could be suitable for developing large scale production. The synthesized Fe nanoparticles were effectively utilized for the antibacterial activity study. The maximum zone of inhibition was found to be against *Pseudomonas aeruginosa*. The metal Nanoparticles were characterized using UV-Vis spectroscopy and FTIR. Biosynthesis of iron nanoparticles using *Annona squamosa* was carried out and the results are interpreted using different analytical instruments like UV-Vis spectrophotometer which indicate the formation of iron nanoparticles at 271 nm, followed by FTIR that was used in identifying the functional groups present in synthesized nanoparticles. Further studies showcased the anti biofilm property of the synthesized Fe nanoparticle solution, *Pseudomonas aeruginosa* by agar well diffusion method. In the near future, the nanocollodis may play major role in the treatment of infections caused due to Multidrug Resistant pathogens biofilm.

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