

Green Synthesis Of Silver Nanoparticles Using *Tinospora Cordifolia* And Its Antibacterial Activity Against Wound Pathogens

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Abstract: Nanotechnology has become an emerging tool of sophisticated strategy in the current scientific era and has become a major contributing factor to the field of therapeutics. Wound infections are one of the most common infections with the ability to cause significant mortality and morbidity. The increase in the emergence of drug resistance has made the researchers to look for an alternative strategy and in this scenario; nanotechnology combined with the traditional medical plants can serve as the best candidate for an alternative regimen to the current chemotherapy. In this study, Pus samples were collected from Hospitals and the pathogens were isolated and identified. Then Silver Nanoparticles were synthesized from *Tinospora cordifolia* and their antibacterial efficacy against wound pathogens was tested after Characterization by employing UV-VIS Spectroscopy, X-Ray Diffraction, FTIR, TEM and EDX. The reports suggested that Silver nanoparticles exhibited potential antibacterial activity against the pathogens like *Pseudomonas aeruginosa* and *Staphylococcus aureus*. In the future, these synthesized nanoparticles of *Tinospora cordifolia* candidates for treatment against pus pathogens

Keywords: *Tinospora cordifolia*, Nanotechnology, UV-VIS Spectroscopy, FTIR, X-Ray Diffraction, TEM, EDX

INTRODUCTION

Wound infection is considered to be one of the most common as well as potentially devastating complications of improper healing of wound. If ignored the wound infection has extreme capabilities to manifest as severe complications at a risk of increased medical expenses, and even cause fatalities (Bessa *et al.*, 2015). A thorough understanding of the pathophysiology of wound infection is very much crucial for the prevention of severe impacts and every medical practitioner engaged in wound care should be equipped with the knowledge of wound infection. Most of the wound infections are caused primarily by the Bacteria and some of the most common causative organism associated comprise of *Staphylococcus aureus*, *Streptococcus pyogenes*, *Klebsiella pneumonia*, *Enterococci faecalis* and *Pseudomonas aeruginosa* (Bowler, 2009).

The extensive misuse of the antibiotics over the counter along with genetic and non-genetic alterations has led to the birth of Antimicrobial Resistance which is a leading health threat in this current scenario (Abdulgader *et al.*, 2021.). The emergence of Drug resistance in Bacteria is difficult challenge in the field of infectious diseases and this has been complicated to an extreme level with the advent of Multidrug resistant strains. Hospital-acquired wound infections causes significant morbidity and

mortality in the Nosocomial case statistics (Barrasa-Villar *et al.*, n.d.). Among the wound infections, infections associated with burns are at an increased risk of acquiring secondary bacterial infections. *Pseudomonas aeruginosa*, a predominant agent behind burn wound infection and is also a subsequent cause of Septicemia and death. The burn injuries are characterized by the disruption of the anatomical barrier such as the normal skin, suppression of systemic host defense mechanism and increased susceptibility of the skin surface to microbial colonization thereby paving path for the development of burn wound sepsis (Church *et al.*, 2006). The application of a novel efficient prophylactic as well as therapeutic strategies is of dire need for the prevention of the wound infections.

The increase in the progressive resistance of microbial pathogens to the traditional antibiotics has become a common phenomenon and in this scenario, the researches on the quest for an alternative strategy that is equipped enough to combat drug resistance is on the rise. In view of this concept, special consideration is being diverted to the field of Nanotechnology and nanoparticle-based materials. The emergence of Nanoparticles with antibacterial properties has given a lead start for the advent of novel therapeutic regimens. Several nanoparticles (NPs) and carriers are available for delivery of antibiotics which has efficacies of therapeutic potential against infections comprising both in vitro as well as in vivo models (Varier *et al.*, 2019). Nanotechnology in the current scenario confers a base for the adjustment of the physiological and chemical properties of various materials for synthesizing therapeutic agents (Lee *et al.*, 2019). Nanomaterials (NM) can be considered as strategically advantageous as their surface area is relative large to their size. Nanosized particles are also equipped with the ability to demonstrate high activity despite being employed in small doses and are excellent candidates that can serve as candidates for controlling infections.

Synthesis of nanoparticles can be Physicochemical or biological in nature and amongst them, the Chemical approaches are considered to be highly efficient (Jamkhande *et al.*, n.d.). Noble metal based nanoparticles such as the gold, silver as well as platinum have extensive application in the manufacturing of nanoparticles. Several types of nanoparticles are available and among them the Metals and metal oxides based nanoparticles extensively evaluation for antimicrobial activities owing to their bactericidal properties which include reactive oxygen species (ROS) generation and metal ion release (Ficai & Grumezescu, 2017). Silver NPs (AgNPs) are considered as the most efficient ones amongst the other metal based nanoparticles and have been reported to show significant activity against the bacteria and viruses. In addition, resistance against Silver is not easily developed in the pathogens as this metal has several target sites in Micro organisms (Loo *et al.*, 2018). AgNPs are capable of targeting respiratory chain as well as the cell-division machinery, with release of silver ions (Ag⁺) which would lead to the enhancement of their bactericidal activity resulting in death of the pathogens (Zhang *et al.*, 2018). The nanoparticles have found diverse applications in dressings for wound, as antimicrobial coating on medical tools and masks, in membranes, and as nanoparticle based gels (El-Kamel *et al.*, 2015).

The employment of plants for the greener manufacturing of AgNPs has gained significance as they are less expensive, conveniently used for bulk synthesis, needs less maintenance and also the efficiency can be improved due to the presence of antioxidants, which serve as reducing and capping agents (Keat *et al.*, 2015). The application of plant source as a medicine has been practiced for several millenniums and in the modern world; scientists have begun to probe into the value of medicinal plants as source of novel therapeutic agents. India has been identified as a country enriched with traditional and alternative medicine (Pandey *et al.*, 2013). The Indian public uses more than 8000 species of medicinal plants and these have been found to possess anti-complementary as well as immunomodulatory activities (Samal, 2016). In this study, *Tinospora cordifolia* has been employed for the investigation of its properties for its employment in Wound care. *Tinospora cordifolia* is a member of *Menispermaceae*

family. It is a large climber present abundantly in India and colloquially mentioned as heart leaved moonseed plant in English, as the *Guiduchi* in Sanskrit and named as Giloy in Hind. It has been established medicinally important plant due to the presence of Tubocurarine, Colchicines, Nicotine and Quinine in it etc. All parts like leaves, stem, fruits and roots are rich in Nutraceuticals (Mittal *et al.*, n.d.). These defensive properties of plant products are due to the presence of macro and micro nutrients, antioxidants and phytochemicals and these properties have enabled the enrollment of *Tinospora cordifolia* for this study

This study explores on the characterization of silver nanoparticles synthesized by the application of the plant extract of *Tinospora cordifolia* along with the analysis of their antibacterial activity, antioxidant potential and cytotoxicity significance AgNps against wound pathogens.

1. MATERIALS AND METHODOLOGY

2.1 Collection of the plant and preparation of plant powder:

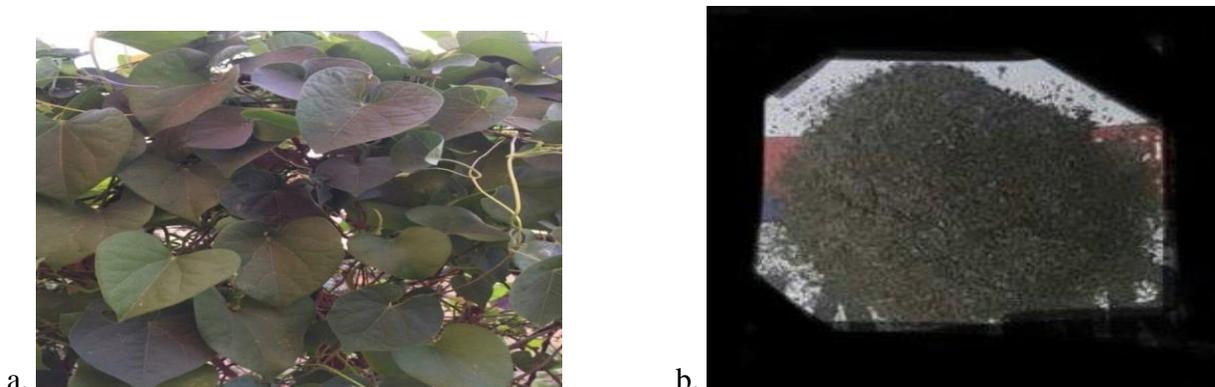


Fig. 1: a. *Tinospora cordifolia* leaves and b. its dried leaf powder

Seenthil (*Tinospora cordifolia*) plant leaves were collected nearby kalavai. It was cleaned from impurities with distilled water, dried in shade and ground to powder form. It was then subjected to storage in air tight containers at room temperature.

2.2 Preparation of plant extract:

2 gm of the *Tinospora cordifolia* powder was weighed using electronic balance and was dissolved in distilled water of approximately 100 ml taken in a clean conical flask. It was then boiled at 60° C for a period of 10 mins. Then plant extract was subjected to filtration employing Whatmann filter paper No.1.



Fig. 2: Extraction of active metabolites from *Tinospora cordifolia* leaf powder

2.3 Isolation and identification of organism from Pus sample:

2.3.1 Sample collection:

The pus samples were collected from nearby hospitals and lab G.B Nagar, Ranipet district. The collected samples were packed in sterile container to avoid contamination. After collection of sample, preliminary process like staining and inoculation in selective media was done.

2.3.2 Gram's staining procedure:

One loop of culture was taken and thin smeared on a clean glass slide. It was then heat fixed and flooded with gram's crystal violet for 1 minute. Rinsing was done with distilled water and gram's iodine was flooded over it for 1 minute. Then it was washed with a gram's decolourizer followed by a wash with distilled water. Then, the counter stain safranin was flooded over the smear for 1 minute. Then washed with distilled water, air dried and was subjected to observation under oil immersion objective.

2.3.3 Catalase test:

A clean loopful of culture was taken in grease free slide. Then it was mixed with a drop of 3% hydrogen peroxide.

2.3.4 Oxidase test:

The oxidase disc was taken on a clean glass slide. Then a drop of broth culture was dropped over the disc.

2.3.5 Biochemical Test:

- **Indole Test:**

5 ml of sterile peptone broth was prepared and inoculated with overnight test culture. Then, subjected to incubation at 37° C for a period of 24 hrs. After the incubation, Kovac's reagent was added.

- **Methyl Red Test:**

5 ml of sterile MR-VP broth was prepared and inoculated with overnight test culture. Then, subjected to incubation at 37° C for 24 hrs. After incubating, 2-3 drops of methyl red indicator was added.

- **Voges-Proskauer Test:**

5 ml of sterile MR-VP broth was prepared and inoculated with culture. The incubated at 37°C for 24 hrs. After incubation, addition of the indicator reagents such as Barrit's reagent A and Barrit's reagent B was done. Then tubes were then shaken to mix well and allowed to stand for 30 minutes for the observation of color change.

- **Citrate Utilization Test:**

5 ml of sterile Simmon's Citrate Agar was prepared and the culture was streaked on the agar slant. Then, incubated at 37°C for 24 hrs.

- **Triple Sugar Iron Test:**

5 ml of triple sugar iron agar was prepared and the culture was stabbed and streaked on the agar slant. Then, incubated at 37°C for 24 hrs.

2.4 Synthesis of Silver Nanoparticles:

80 ml of 1 mM of AgNO₃ solution and 20ml of plant extract were taken in a labelled and sterile conical flask. The conical flask solution was mixed well and then kept in rotator shaker for 3 days. The reaction in the mixture was observed for color changes at an interval of 2 hrs. The color changes were then analyzed using UV-Vis Spectrophotometer. After completion of the reaction, the synthesized (AgNO₃) were harvested by centrifuging at 8000 RPM for 10 mins. After centrifugation the pellets were rinsed with distilled water and dried overnight. Then the powdered forms of pellet were collected in clean vials and used for characterization and identification.



Fig 3: Synthesis of Nanoparticles

2.5 Characterization of Nanoparticle:

Characterization of the AgNPs was carried by subjecting them to UV Visible Spectrophotometer Analysis, Transmission Electron Microscopy, X-Ray Diffraction Assay (XRD), EDX (Energy Dispersive X-Ray) and Fourier Transform Infrared Spectroscopy (FTIR):

2.6 Antibacterial Activity:

The bacterial broth culture was swabbed onto sterile Mueller Hinton Agar plated. Then four wells were cut using gel puncture. The first three wells were added with sample and antibiotic disc was placed in the fourth well. Then the plates were subjected to incubation at 37°C for period of 24 hrs. After incubating, the zones were measured and the readings were tabulated.

2.7 Antioxidant Test:

29.5ml of methanol and 0.5µl of DPPH were mixed well. Synthesized plant materials was taken in 10µl, 20µl, 30µl, 40µl, 50µl concentration. All these test tubes were added with 1ml of previously prepared solution and then kept in dark place for incubation for 10 mins. Readings were observed in UV Spectrophotometer.

2.8 Cytotoxic Activity:

3000ml of water was taken in rectangular jar. 27 gram of common salt was weighed using balance and then added into the jar and mixed well, then the tip of an air line of air pump was placed at bottom for aerating, 15 g of brine shrimp eggs were then added to the top and then mixed with water, a light source (60-100 Watt bulb) was provided a few inches away from the jar. After 24 hrs, the eggs were observed for nauplii hatching. Then 10 nauplii were transferred to test tube. The nauplii were exposed to various concentration of the extract and then survivors were calculated and death percentage was recorded after a period of 24 hours.

2. RESULTS

3.1 Isolation and identification of organism from Pus sample:

The Figures 4-8 and Table 1-3 represents the different organism identified from the clinical sample.

Table 1: Preliminary identification of isolates from clinical sample

Organism	Gram staining	Capsule Staining	Spore Staining	Motility test	Catalase test	Oxidase test
<i>Staphylococcus Sp</i>	Gram Positive cocci	Non capsulated	Non spore Negative	Non motile	Positive	Negative

<i>Streptococcus Sp</i>	Gram Positive cocci	Capsulated	Non spore Negative	Non motile	Positive	Negative
<i>Klebsiella Sp</i>	Gram negative short rod	Capsulated	Non spore Negative	Non motile	Positive	Negative
<i>Enterococcus Sp</i>	Gram Positive cocci	Non capsulated	Non spore Negative	Non motile	Negative	Negative
<i>Pseudomonas Sp</i>	Gram Negative rod	Non capsulated	Non spore Negative	Motile	Positive	Positive

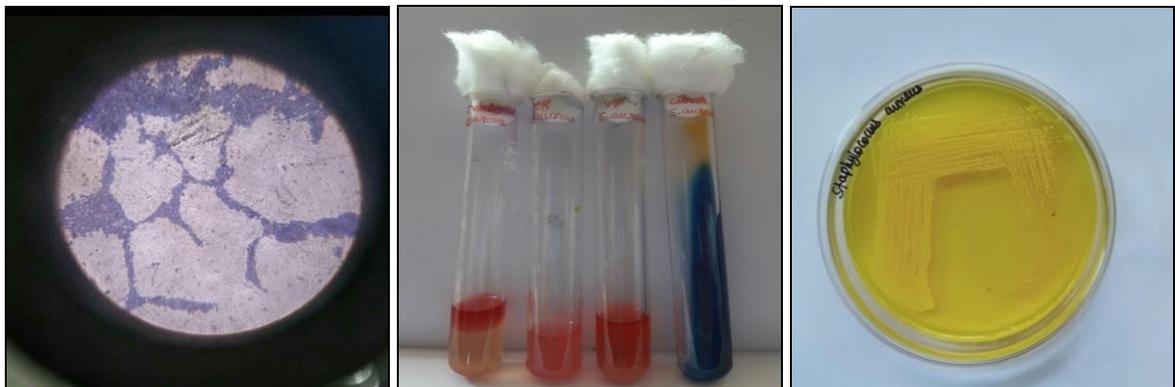


Fig. 4. Identification of *Staphylococcus Sp*



Fig. 5. Identification of *Streptococcus Sp*

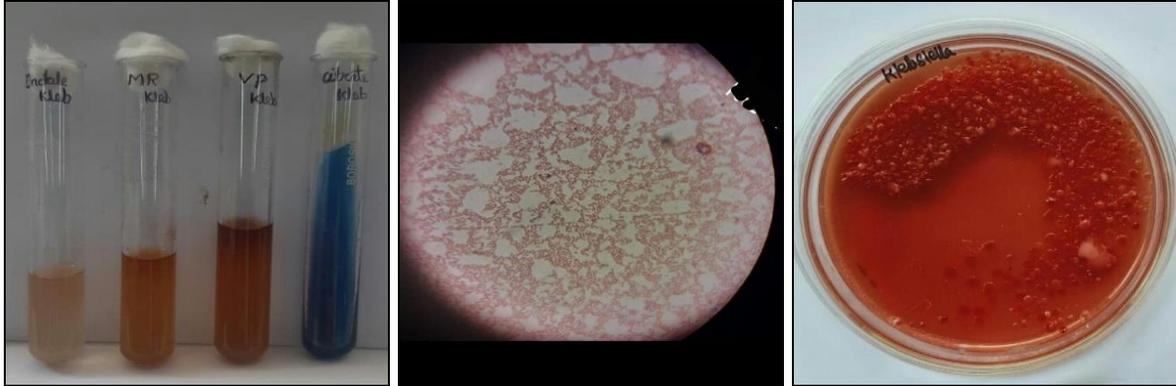


Fig. 6. Identification of *Klebsiella Sp*

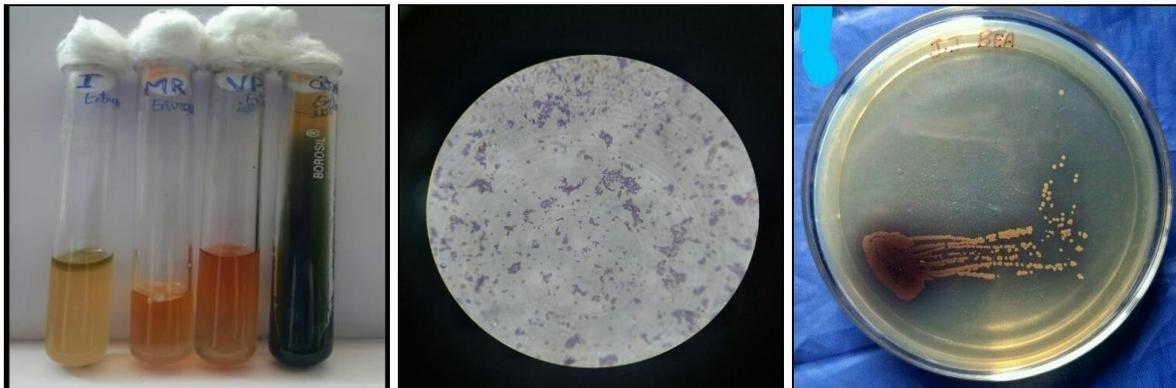


Fig. 7. Identification of *Enterococcus Sp*

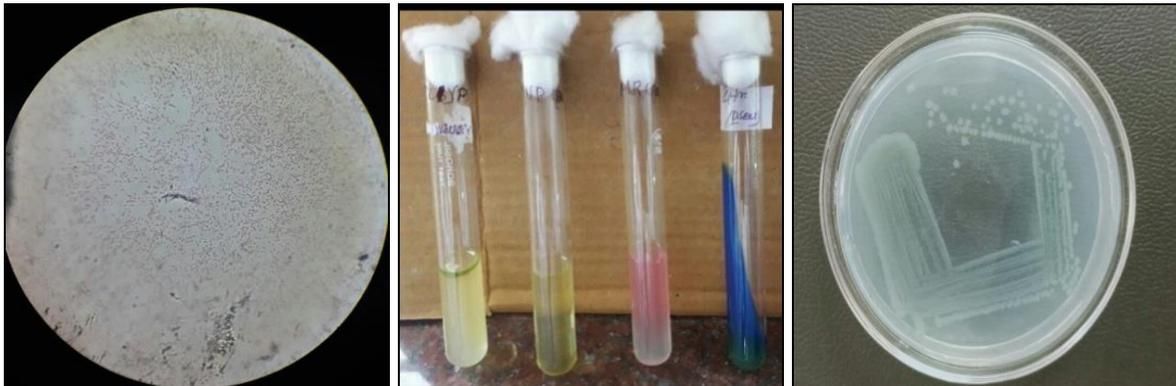


Fig. 8. Identification of *Pseudomonas Sp*

MEDIA	ORGANISM
MANNITOL SALT AGAR PLATE	Golden yellow colonies of <i>Staphylococcus Sp.</i>
TRYPTIC SOY AGAR PLATE	White greyish colonies of <i>Streptococcus Sp.</i>
MACCONKEY AGAR PLATE:	Pink mucoid colonies of <i>Klebsiella Sp.</i>
BILE ESCULIN AGAR PLATE:	White colonies of <i>Enterococcus Sp.</i>
CETRIMIDE AGAR PLATE:	Green pigmented swarming growth of <i>Pseudomonas Sp.</i>

Table2: Colony morphology of isolates from clinical sample

ORGANISM	INDOLE	MR	VP	CITRATE	CATALASE	OXIDASE
<i>Staphylococcus Sp</i>	Positive	Positive	Positive	Positive	Positive	Negative
<i>Streptococcus Sp</i>	Negative	Positive	Positive	Positive	Positive	Negative
<i>Klebsiella Sp</i>	Negative	Negative	Positive	Positive	Positive	Negative
<i>Enterococcus Sp</i>	Negative	Negative	Positive	Negative	Negative	Negative
<i>Pseudomonas Sp</i>	Negative	Negative	Negative	Positive	Positive	Positive

Table3: Biochemical identification of isolates from clinical sample

2.2 UV- Visible spectroscopic analysis:

The analysis obtained by UV-Visible spectroscopy has been represented in the Fig 9

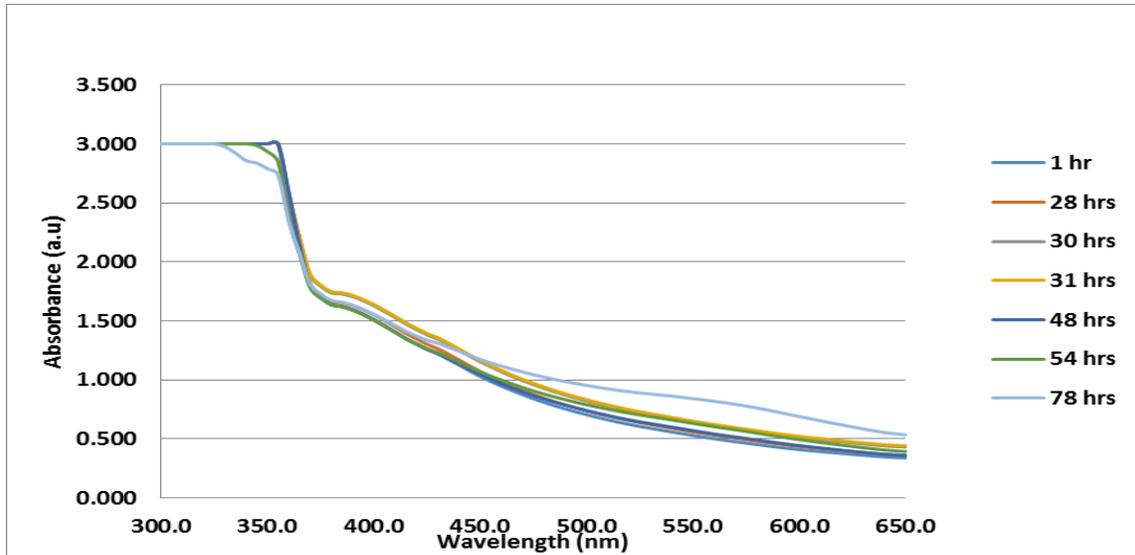


Figure 9: UV-Vis spectroscopic analysis of silver nanoparticles

3.3 Transmission electron microscopic analysis

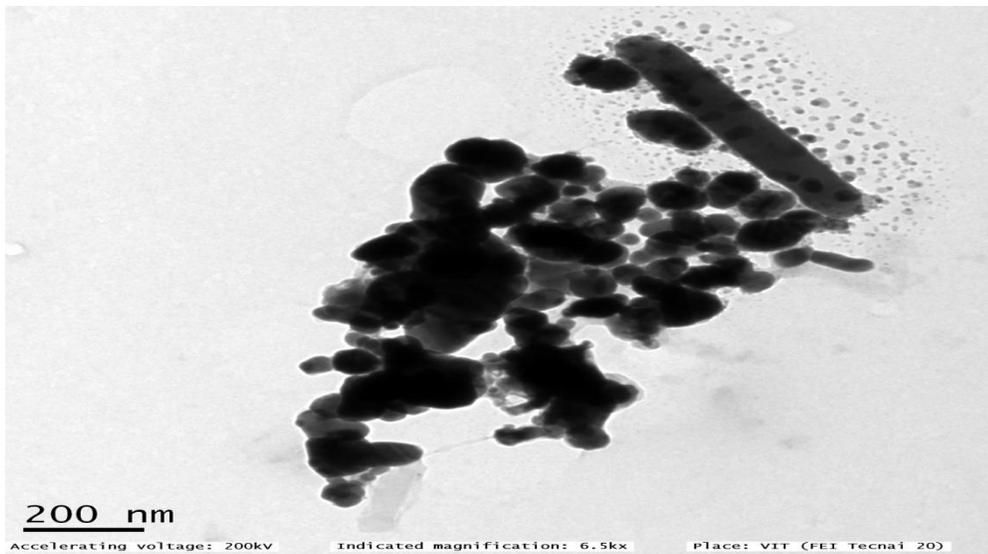


Figure 10: TEM analysis of silver nanoparticles

The structural analysis synthesized NPs obtained has been represented in the Fig 10. The figure 8 represents the transmission electron microscopic image of the silver nanoparticles synthesized using *Tinospora cardifolia* extract. The spherical and rectangle shaped nanoparticles are represented in the image. The size was in 8 – 74 nm range with many agglomerations due to the presence of plant extract.

3.4 Fourier transform infrared spectroscopy:

The Fourier transform infrared spectroscopic analysis of the obtained has been represented in the Fig 9

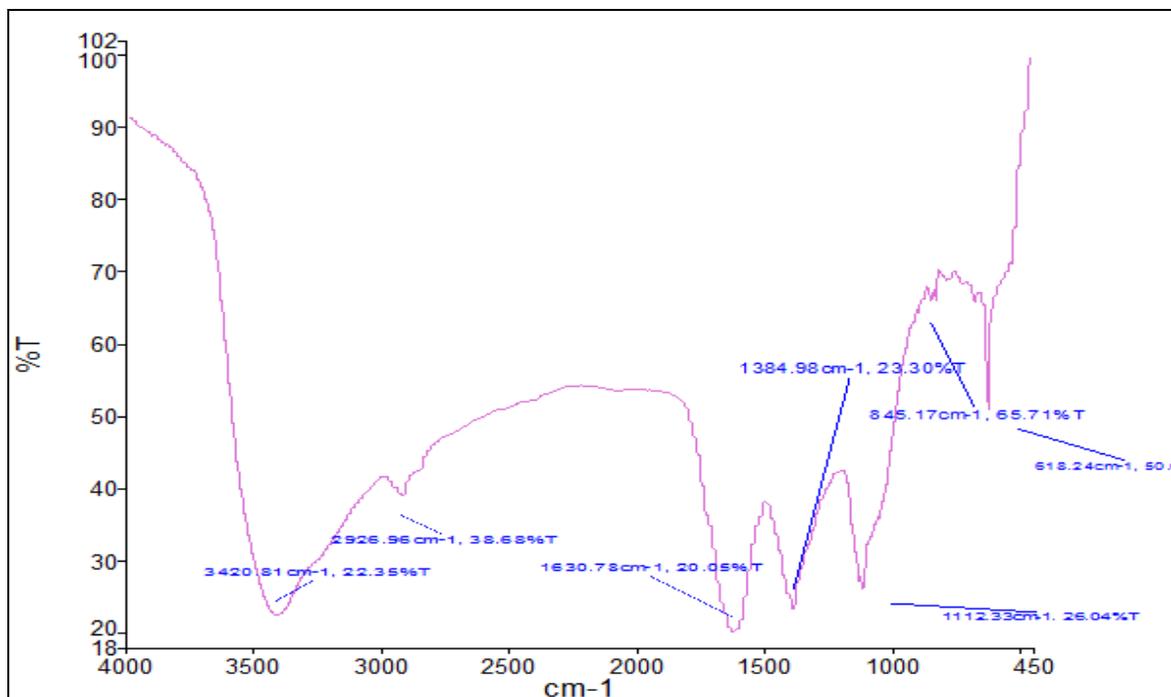


Figure 11: FTIR analysis of silver nanoparticles

3.5 Antioxidant Test:

The antioxidant activity is represented in the figure 12.



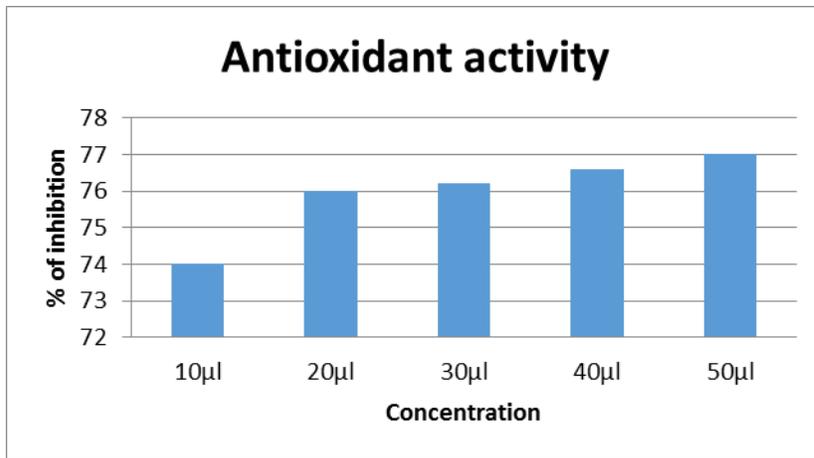


Fig 12: Antioxidant activity of synthesized NPs.

3.6 Cytotoxic Activity:

The cytotoxic activity is represented in the figure 13.

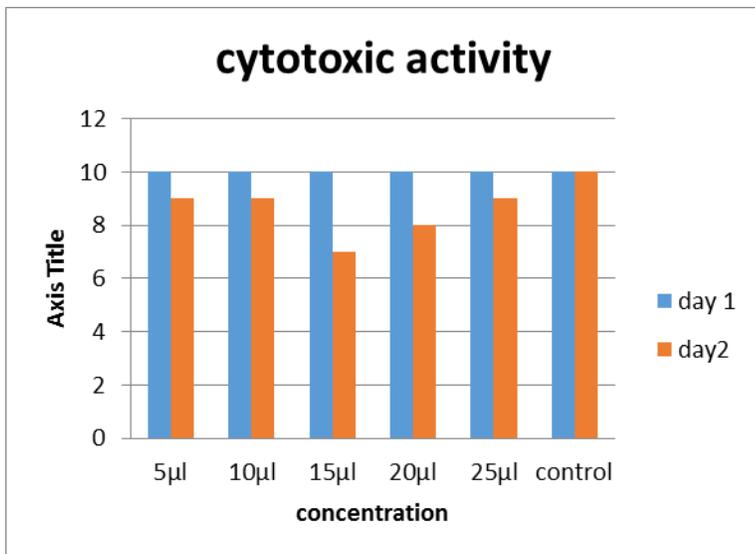
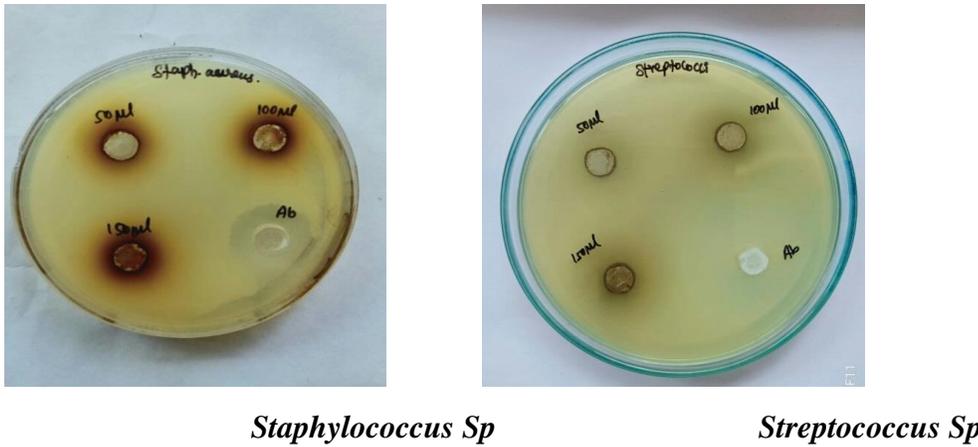


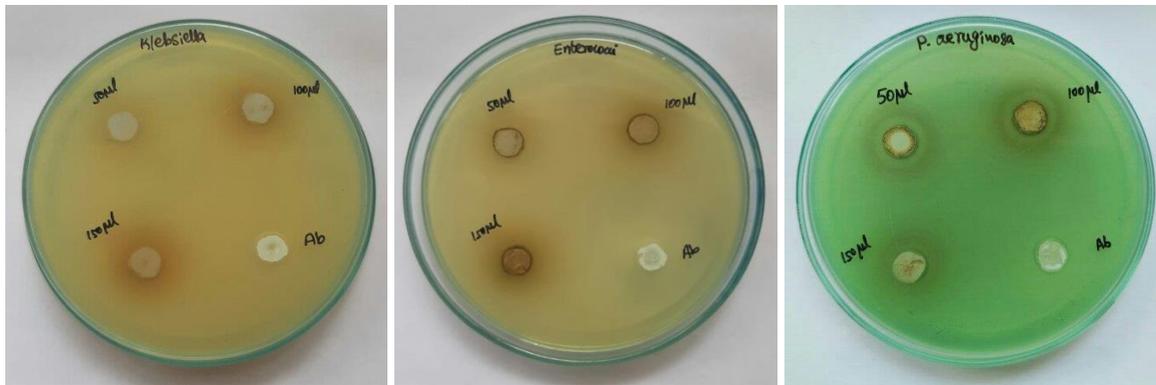


Fig 13: Cytotoxic activity of synthesized NPs.

3.7 Antibacterial assay:

The results of the Antibacterial assay have been represented in the figure 14.





Klebsiella Sp

Enterococcus Sp

Pseudomonas Sp

Fig. 14. Antibacterial profiling

3. DISCUSSION:

Nanoparticles are building blocks of nanotechnology and are known for exhibiting tremendous characteristic features which are based on their size and morphological properties (Khan *et al.*, n.d.). Nanotechnology is considered to be the most advanced field of study in modern science (Schummer, 2004). Several noble metals like silver, gold and platinum have been known to exhibit broad range of behavior (Sreeprasad *et al.*, 2013). Among other metallic nanoparticles, silver has found extensive applications in field of dentistry as well as a health additive in the traditional Chinese and Indian medicine (Journal, Satpathy, *et al.*, 2018). Silver nanoparticles exhibit tremendous applications in drug delivery, sensor applications (Singh *et al.*, 2008) wound healing (Journal, Mathur, *et al.*, 2018) and are also employed as antimicrobial agent (Rath *et al.*, 2016). Silver nanoparticles are renowned for their antimicrobial actions in food pathogens such as *Staphylococcus aureus* and *Escherichia coli* (Zorraquín-Peña *et al.*, 2020), *Pseudomonas aeruginosa* and *Klebsiella pneumonia* (Ibrahim, 2019), *Streptococcus pyogenes* and *Salmonella typhi*. Biological synthesis of silver nanoparticles by employing microbial enzymes, algae as well as extracts of plants possess higher advantages as they are environment friendly and can be easily scaled up bulk production when compared to the conventionally available physical and chemical techniques. Biological synthesis has slower kinetics, enhanced manipulation and stabilization. Green synthesis of silver nanoparticles is more advantageous over other biological processes than bacterial and fungal based method because it eliminates the cell culture maintenance (Ibrahim, 2019).

Our studies were found to be consistent with another study Samir *et al.*, 2013, Silver nanoparticles from stem powder extracts of *Tinospora cordifolia* have been known to have bactericidal and inhibitory effects and were effective against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* (Anuj, 2019). In another study by Selvam *et al.*, 2016, Silver nanoparticles synthesized using extracts of *Tinospora cordifolia* have been known to possess bactericidal and bacteriostatic effects and were effective against *Staphylococcus aureus* and *Klebsiella sp.* (Selvam *et al.*). This study is in concordance with our outcomes. In the study by Gopinath *et al.*, 2012, AgNpS were synthesized from *Tribulus terrestris*, the efficiency against the synthesized nanoparticles was analysed by Kirby–Bauer method with clinically isolated multi-drug resistant bacterial strains comprised of the *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Escherichia coli* and *Staphylococcus aureus* (Gopinath *et al.*, n.d.). This supports the ideology of our study which is aimed in identifying a suitable candidate of efficiency for therapeutic use in wound care. The extract based silver nanoparticles are

synthesis relatively rapid and less expensive with the benefit of wide application in antibacterial therapy and are excellent alternatives to the conventional chemotherapy administered.

4. CONCLUSION:

The Greener synthesis technology of nanoparticles provides several advantages over several conventionally employed physical and chemical method owing to its cost effectiveness, environment friendly properties and ability to be conveniently scaled up for large scale synthesis. Moreover, this technique doesn't require high pressure, energy or toxic chemical which altogether makes it a potential strategy for Nanoparticle synthesis. In this study, the extract of *Tinospora cardifolia* was employed in the synthesis and Silver Nanoparticles. The synthesized nanoparticles were then subjected to characterization and were evaluated for their cytotoxic ability and antibacterial properties, the Silver nanoparticles demonstrated significant antibacterial efficacy against *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Hence by employment of the traditionally acknowledged medicinal plants, the efficacies of nanoparticles in the field of therapeutics can be improved.

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