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GREEN SYNTHESIS OF SILVER NANOPARTICLES USING JUSTICIA ADHATODA AND ITS ACTIVITY AGAINST SELECTED CLINICAL ISOLATES

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ABSTRACT

Nanotechnology deals with the synthesis of nanoparticles using living organisms. Plant has been used for synthesis of metal nanoparticles. Most of the reported green synthesis method using plant took more than 1hour for the formation of colloidal silver. Plant use can also be suitable for large scale synthesis of nanoparticles. *Justicia adhatoda* is one of the important herbs comparising several medical properties. About ten samples of pus, fifteen samples of urine, ten samples of throat and ten samples of wound where suspected growth was appeared in two samples of pus(A4 and A7), three samples of urine(B8 and B11), one sample of throat(C4), two samples of wound(D5 and D7). Based on the morphological and biochemical characteristics the organisms were identified as *Staphylococcus aureus (*A4, B11), *Enterococcus faecalis* (B8, D7), *Pseudomonas aeruginosa*(A7,D5), *Micrococcus luteus(C4).* The plant *J. adhatoda leaves* were collected from Ramanathapuram (Dt). The washed leaves were shade dried for 7 days. The dried plant leaves were then ground into powder. The finely ground *Justicia adhatoda* powder(10g) material was mixed with 100 ml of distilled water and kept in boiling at 100^{o} C for 20 minutes. It was filtered with help of whatmann no: 1 filter paper and then stored in room temperature. 1mM and 2mM silver nitrate were prepared. Reduction of Ag+ ions was monitored by measuring the uv- vis spectrum. Absorption spectra of silvernanoparticles formed in the reaction media has the absorbance peak at 434nm. SEM analysis was done. SEM image also showed spherical shaped nanoparticle that were formed with a diameter ranges 84nm. Xray diffraction studies confirmed crystalline nature of the silver nanoparticles. The crystallite domain size was calculated by using the width in the XRD peaks. The peak

value showed 38.3°, 46.37°, 64.53° and 77.40° corresponding to (111), (200) and (31) respectively, when compared to the XRD spectrum in the standard. Biosynthesis of silver nanoparticles were studied for antimicrobial activity against selective human pathogenic bacteria by using standard zone of inhibition (ZOI) microbiology assay, with a well size of 5 mm diameter and 20 µl of samples. Reference drug l of 0.1mg/ml concentration was used as a control antibacterial agent. The silver nanoparticles synthesized showed inhibition zone against all the isolates like *Staphylococcus aureus (*A4, B11), *Enterococcus faecalis* (B8, D7), *Pseudomonas aeruginosa*(A7, D5), *Micrococcus luteus(C4).*

KEYWORDS: *Justicia adhatoda*, Silver nanoparticles, XRD, Nanoparticles, SEM.

INTRODUCTION

Nanotechnology is one of the most interesting areas which are used to describe the creation and utilization of materials with structure features between those of atom and bulk materials with at least one dimension in the nanorange (Saxena *et al., 2012).*[1] Nanotechnology deals with the synthesis of nanoparticles using living organisms. Plants have been used for synthesis of metal nanoparticles. Nanotechnology permits broad advances in agricultural research, such as reproductive science and technology, conversion of agricultural and food wastes to energy and other useful by-products through enzymatic nanobioprocessing, disease prevention and treatment in plants using various nanocides (Carmen *et al*., 2003).[2]

Nanomaterials are at the leading edge of the rapidly developing field of nanotechnology. The development of reliable experimental protocols for the synthesis of nanomaterials over a range of chemical compositions, sizes, and high mono disparity is one of the challenging issues in current nanotechnology. Recent studies on the use of microorganisms in the synthesis of nanoparticles are a relatively new and exciting area of research with considerable potential for development. Metal nanoparticles are intensely studied due to their unique optical, electrical, and catalytic properties. To utilize and optimize chemical (or) physical properties of nano-size metal particles, large spectrums of research have been focused to control the size and shape, which is crucial in tuning their physical, chemical and optical properties (Bruchez *et al.*, 1998). [3]

Particularly, Silver and Gold nanoparticles have been extensively investigated because of their applications in opto-electronic devices. Recently there have been growing interests in a variety of metal ceramic and metal-dielectric, composite nanoparticles for their potential

technical application. Among silver's many applications, its disinfectant property is being exploited for hygienic and medicinal purposes, such as treatment of mental illness, nicotine addiction and infectious disease like syphilis and gonorrhoea (Adnan *et al.*, 2010).[4] Silver components have been proven as an effective tool for retorting and preventing the bacterial infection and they also exhibit wound healing activity. In addition, silver is known to exhibit oligodynamic effect because of its ability to exert bacterial activity at minute concentration (Tien *et al.*, 2009). [5]

Biological methods of synthesis have paved way for the "green synthesis" of nanoparticles and these have proven to be better method due to slower kinetics, they offer better manipulation and control over crystal growth and their stabilization. The use of environmentally benign materials like plant extract (Jain *et al.*,2009),[6] fungi (Verma *et al.*,2010)[7] and enzymes (Willner *et al.*,2007)[8] for synthesis of silver nanoparticles offer numerous benefits of eco friendliness and compatibility for pharmaceutical and other biomedical applications as they do not use toxic chemicals for the synthesis nanoparticles methods.

Chemical synthesis methods lead to presence of some toxic chemical absorbed on the surface that may have adverse effect in the medical application. Among the use of living organisms for nanoparticle synthesis, plants have found application particularly in metal nanoparticle synthesis. Use of plants for synthesis of nanoparticles could be advantageous over other environmentally benign biological processes (Kumar *et al.*, 2009). [9]

Justicia adhatoda is an evergreen greganous stiff perennial shrub. *J. adhatoda* commonly known as *vasaka* distributed throughout India up to an altitude of 1300m. Plants have played a critical role in maintaining human health and civilizing the quality of human life for thousands of years. The use of plants as medicines is a sold as human civilization itself and out of about 258,650species of higher plants reported from the world; more than 10% are used to cure ailing communities (Shinwari, 2010).[`10]

Justicia adhatoda is a well known plant drug in Ayurvedic and Unani medicines (Claeson *et al*.0, 2000). It is used by Ayurvedic physicians and possesses some medicinal properties. It has been used for the treatment of various diseases and disorders, particularly for the respiratory tract ailments. The leaves, flowers, fruits, and roots are extensively used for

treating cold, cough, whooping cough, chronic bronchitis and asthma. It is act as a sedative – expectorant, antispasmodic and anthelmintic (Karthikeyan *et al*., 2009).[11]

This plant is a source of Vitamin C and has medicinal uses, mainly antispasmodic, fever reducer, anti-inflammatory agent, anti-bleeding agent, bronchodilator, anti-diabetic agent, disinfectant agent, anti-jaundice and oxytocic agent (Maurya,2010).[12] It is anti periodic, astringent, diuretic, purgative and is also used as an expectorant in addition to liquefy sputum (Salalamp *et al.,* 1996).[13] Leaves are used for curing bleeding, haemorrhage, skin diseases, wounds, head ache and leprosy in Southeast Asia. Adhatoda possesses many remedial measures, in the present investigation it has been selected for the synthesis of silver nanoparticles.

MATERIALS AND METHODS

About 10 samples of pus, 15 samples of urine, 10 Throat samples and 10 wound samples were collected from a private hospital in Chennai. The samples were collected in a sterile container and were transferred to the laboratory for processing. The pathogenic organisms were isolated and purified. They were characterized by various biochemical tests(Monica Cheesebrough).[14]

Plant Material

The plant *J. adhatoda leaves* were collected from Ramanathapuram (Dt). The mature, undamaged and disease free leaves were selected and washed thoroughly with distilled water 2-3 times under laminar air flow chamber. The washed leaves were shade dried for 7 days. The dried plant leaves were then ground into powder, stored in dark glass bottles and kept at low temperatures until further analyses.

Preparation of leaf extract

The finely ground *Justicia adhatoda* powder(10g) material was mixed with 100 ml of distilled water and kept in boiling at 100^{o} C for 20 minutes. It was filtered with help of whatmann no: 1 filter paper and then stored in room temperature (Arunachalam *et al*., 2011).[15]

Synthesis of silver nanoparticles

1mM and 2mM aqueous solution of silver nitrate (AgNO3) were prepared (Nagati *et al.,* 2012) [16] and used for the synthesis of silver nanoparticles. 20 ml of leaf extract was added

into 80ml of aqueous solution of 1mM silver nitrate for reduction into Ag+ ions and kept at room temperature for 5 hours. The reduction of pure Ag+ ions was monitored by measuring the UV-Vis spectrum of the reaction medium at 3 hours after diluting a small aliquot of the sample into distilled water. UV-Vis spectral analysis was done by using UV-Vis spectrophotometer.

The silver nanoparticle solution thus obtained was purified by repeated centrifugation at 5000 rpm for 20 min followed by redispersion of the pellet of silver nanoparticles into 10 ml of deionized water. After freeze drying of the purified silver particles, the structure and composition were analyzed by XRD and SEM. The dried mixture of silver nanoparticles was collected for the determination of the formation of Ag nanoparticles by an X'Pert Pro x-ray diffractometer (PAN alytical BV, The Netherlands) operated at a voltage of 40 kV and a current of 30 mA with Cu Kα radiation in θ-2θ configurations. The crystallite domain size was calculated from the width of the XRD peaks, assuming that they are free from non-uniform strains, using the Scherrer formula.

$$D= 0.94 \lambda / \beta \text{ Cos } \theta$$

Scanning Electron Microscopic (SEM) analysis was done using Hitachi S-4500 SEM machine.

Antimicrobial assay

The antibacterial activity of reduced silver nanoparticles was determined by agar well diffusion method for the isolated cultures (Kora *et al.*, 2009).[17] Bacterial suspension was prepared by growing a single colony overnight in nutrient broth and by adjusting the turbidity to 0.05 McFarland standards. Muller Hinton agar (MHA) plates were spread with 0.1 ml (10^5 to 10^6 bacterial per ml) of this bacterial suspension. 5mm diameter of 4 wells were cut on the plates using well puncture and then 20µl of plant extract, 20µl of 1mM AgNps solution, 20µl of 2mM AgNps solution and 20µl of positive control drug chloramphenicol were added in respective wells. The plates were incubated at 37^o C for 24 hours and the zone of inhibition (ZOI) was measured by subtracting the well diameter from the total inhibition zone diameter. Three independent experiments were carried out with each strain.

Chemicals

Silver nitrate was purchased from Sigma – Aldrich, Bangalore, India. The bacteriological media purchased from HiMedia Laboratories, India. All media and solutions were prepared in

double distilled Milli Q water. The experiment were performed in triplicates and mean values are presented in results.

RESULTS

Among ten samples of pus, fifteen samples of urine, ten samples of throat and ten samples of wound suspected growth was appeared in two samples of pus(A4 and A7), two samples of urine(B8 and B11), one sample of throat(C4), two samples of wound(D5 and D7). The results are shown in table-1. Based on the morphology, cultural characteristics, and biochemical tests, the isolates were identified as Sample A4: *Staphylococcus aureus* Sample A7: *Pseudomonas aeruginosa* Sample B8: *Eterococcus faecalis* Sample B11: *Staphylococcus aureus* Sample C4: *Micrococcus luteus* Sample D5: *Pseudomonas aeruginosa* and Sample D7: *Eterococcus faecalis*

Table.1: **NUMBER OF THE ISOLATES FROM SAMPLES**

NO OF SAMPLES	PUS SAMPLE	URINE SAMPLE	THROAT SAMPLE	WOUND SAMPLE
Pseudomonas aeruginosa	**A7**			**D5**
Enterococcus faecalis		**B8**		**D7**
Staphylococcus aureus	**A4**	**B11**		
Micrococcus luteus			**C4**	

UV-VIS Spectra analysis

It is well known that silver nanoparticles exhibit yellowish brown color in aqueous solution due to excitation of surface plasmon vibrations in silver nanoparticles. The *Justicia adhatoda* dried leaf extract was mixed with aqueous solution of the silver nitrate, it started to change the color from watery to brown due to reduction of silver ion; which indicated the formation of silver nanoparticles. It is generally recognized that UV–Vis spectroscopy could be used to examine size and shape-controlled nanoparticles in aqueous suspensions. Fig. 1 shows the UV–Vis spectra recorded from the reaction medium. Absorption spectra of silver nanoparticles formed in the reaction media has absorbance peak at 434 nm and broadening of peak indicated that the particles were polydispersed.

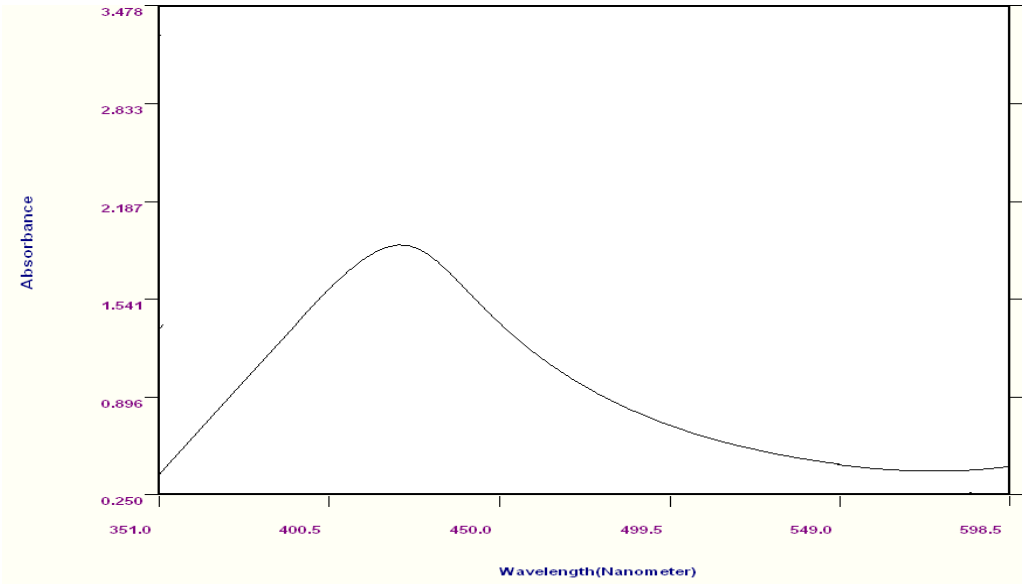
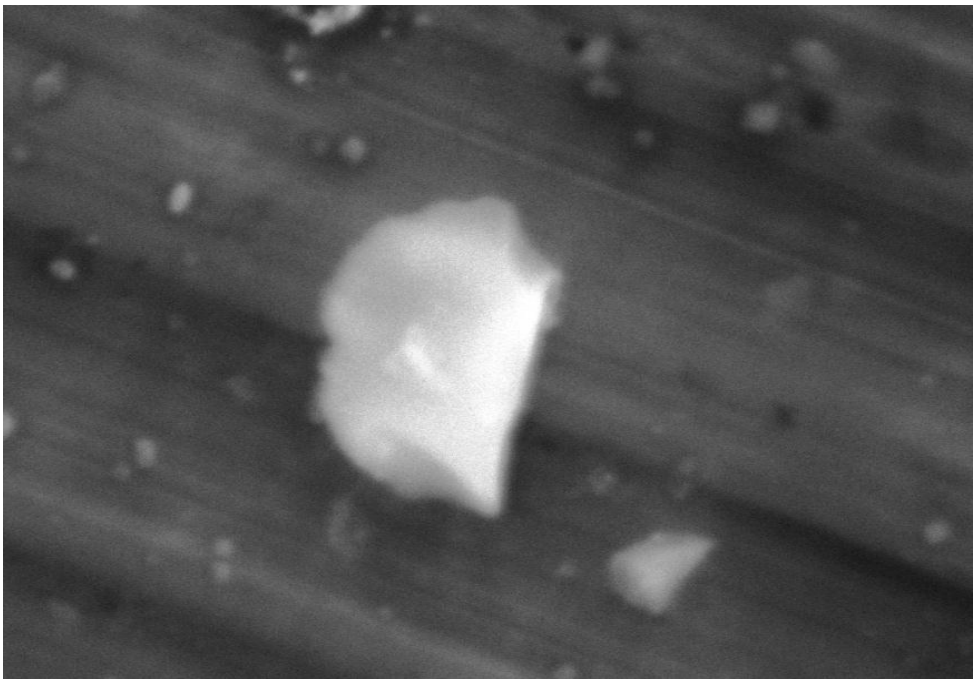


Fig 1.UV–visible absorption spectra of reduction of silver ions to silver nanoparticles

The silver nanoparticles formed were predominantly cubical with uniform shape. It is known that the shape of metal nanoparticles considerably changed their optical and electronic properties. The SEM image showed relatively spherical shape nanoparticle formed with diameter range 84 nm (Fig.2).



XRD Studies

Fig 2. SEM image of Ag nanoparticles formed by *Justicia adhatoda*

Further studies using X-ray diffraction were carried out to confirm the crystalline nature of the particles, and the XRD pattern obtained in Fig. 3 shows number of Braggs reflections that may be indexed on the basis of the face centered cubic structure of silver. There are three intense peaks in the whole spectrum of 2θ values ranging from 24 to 90. A comparison of our XRD spectrum with the Standard confirmed that the silver particles formed in our experiments were in the form of nanocrystals, as evidenced by the peaks at 2θ values of 38.31°, 46.37°, 64.53° and 77.40°, corresponding to (111), (200), (220) and (311), respectively, for silver. These results clearly show that the silver nanoparticles formed by the reduction of Ag+ ions by the *Justicia adhatoda* dried leaf extract are crystalline in nature. As mentioned in the method section, the silver nanoparticles once formed were repeatedly centrifuged and redispersed in sterile distilled water prior to XRD analysis, thus ruling out the presence of any free compound/protein that might independently crystallize and give rise to Bragg reflections.

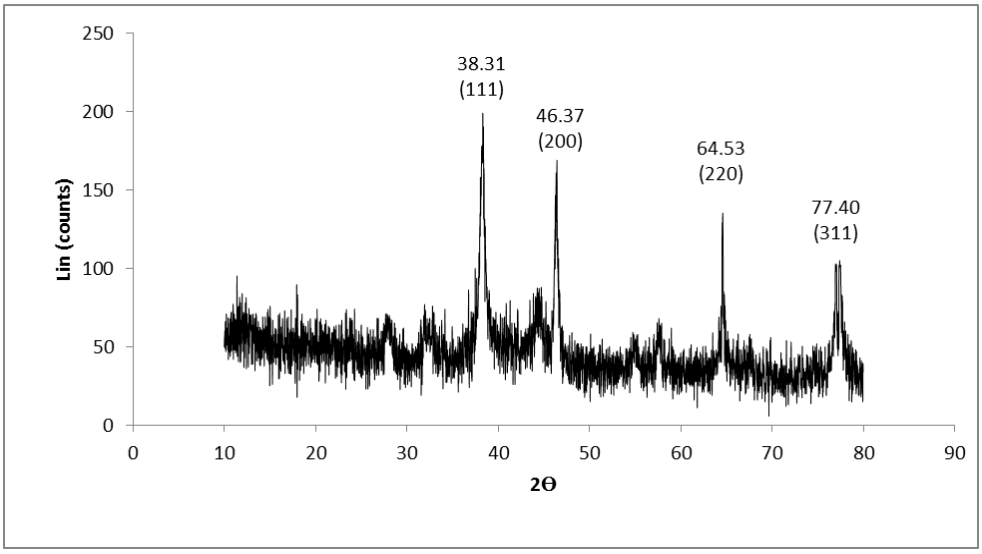


Fig 3.XRD patterns recorded from drop-coated films on glass substrate of silver nanoparticles synthesized by treating *Justicia adhatoda* leaf extract with AgNO3 aqueous solutions. The Bragg reflections are indexed on the basis of the silver structure.

Antibacterial activity

The antibacterial activity highest for *Pseudomonas aeruginosa* was 10±1.5mm and 8.5±1.9mm for *Enterococcus faecalis* at 1mM AgNPs 20µl. 2mM AgNPs gave highest activity (8.3±2.6mm) for *Enterococcus faecalis* followed by 8±1.2mm for *Pseudomonas aeruginosa*. (Table.2)

Table-2 The antibacterial activity of silver nanoparticle synthesis from Justicia *adhatoda*.

isolated human bacterial pathogens	Zone of inhibition in mm			
	Leaf extract 20µl	1mM AgNPs 20µl	2mM AgNPs 20µl	Chloramphenicol (positive control)20µl
Staphylococcus aureus A4(pus)	3±1.6	7±1.3	4±1.6	10±1.3
Pseudomonas aeruginosa A7(pus)	4±1.5	10±1.5	8±1.2	12±1.4
Enterococcus faecalis B8(urine)	4±1.6	8.5±1.9	8.3±2.6	8±0.7
Staphylococcus aureus B11(urine)	3±1.7	8±2.3	7±2.1	10±0.9
Micrococcus luteus C4(throat)	2±0.7	7±1.9	5±2.1	10±1.8
Pseudomonas aeruginosa D5(wound)	3±0.5	10±2.4	9±1.7	12±2.8
Enterococcus faecalis D7(wound)	3.8±1.7	7±0.4	3±1.5	10±0.6

Mean values of triplicate (zone of inhibition) ± S.D.

DISCUSSION

Silver has been widely utilized for thousands of years in human history; its applications include jewels, utensils, currency, dental alloy, photography and explosives. Among silver's many applications, its disinfectant property is being exploited for hygienic and medicinal purposes, such as treatment of mental illness, nicotine addiction and infectious disease like syphilis and gonorrhea (Gulbrason, 2000). [18] Silver nanoparticles have been demonstrated to exhibit antimicrobial properties against bacteria (Sondi and Salopek-Sondi, 2004) [19] with close attachment of the nanoparticles themselves with the microbial cell and the activity being size dependent.

Silver has long been recognized as having inhibitory effect on bacterial strain and other microorganisms present in medical and industrial process. The present study deals with the synthesis of silver nanoparticles using dried leaf extract of *Justicia adhatoda* and aqueous Ag+ ions. Comparative experiments were carried out to study the effect of biomass dosage and the concentration of silver nitrate on the rate of bio reduction of silver ions. The approach

appears to be cost effective alternative to conventional methods of assembling silver nanoparticles. During the formation of nanoparticles, the aqueous silver iron when exposed to herbal extracts was reduced in the solution, thereby leading to the formation of silver hydrosol. As the extract was mixed in the aqueous solution of the silver ion complex, it started to change the color from watery to yellowish brown due to reduction of silver ion which indicated the formation of silver nanoparticles. The same method was followed by Lingarao (2012). [[20]] Ahmed *et al.*, (2011) [[21]] mentioned three different routes for the reduction of silver in plant extracts.

In the present study the synthesis of silver nanoparticles was confirmed by spectroscopy analysis. It is generally recognized that UV–Vis spectroscopy could be used to examine size and shape-controlled nanoparticles in aqueous suspensions. Absorption spectra of silver nanoparticles formed in the reaction media has absorbance peak at 434 nm and broadening of peak indicated that the particles were polydispersed. SEM images Fig 2 recorded from drop-coated films of the silver nanoparticles synthesized by treating silver nitrate solution with dried *Justicia adhatoda* leaf extract. The SEM image in the present study showed relatively spherical shape nanoparticle formed with diameter range 84 nm. This is similar to the reporte of Chandran *et al.*, (2006). [[22]] X-ray diffraction was carried out to confirm the crystalline nature of the particles, and the XRD pattern obtained. There are three intense peaks in the whole spectrum of 2θ values ranging from 24 to 90. A comparison of our XRD spectrum with the Standard confirmed that the silver particles formed in present experiments were in the form of nanocrystals, as evidenced by the peaks at 2θ values of 38.31°, 46.37°, 64.53° and 77.40°. These results clearly indicate that the silver nanoparticles formed by the reduction of Ag+ ions by the *Justicia adhatoda* dried leaf extract are crystalline in nature. This is correlated with the report of Huang *et al*., 2007. [[23]]

Antimicrobial activity

Silver is well known as one of the most widespread antimicrobial substances. According to Sushmita Deb, *(*2014) [[24]] submit the silver nano particles were found to be superior against *vibrio cholera* strain using the well diffusion method, showing 20.5±3.7mm of inhibition zone. Antimicrobial effect of silver nanoparticles obeyed a dual action mechanism of antimicrobial activity, (i.e.) the bactericidal effects of Ag+ and membrane disturbing effects of the polymer subunit. In present study biosynthesis of silver nanoparticles was studied for its antimicrobial activity against the selected human bacterial pathogens by using well

diffusion method (ZOI) was measured by mm diameter by using the reference drug of chloromphenicol 0.1mg/ml concentration was used as a control for antibacterial agent.

Charusheela *et al.*, (2009) [25] reported the synthesis of antibacterial activity of silver nanoparticles (AgNPs) using leaf broth of medicinal herb, *Ocimum Sanctum (Tulsi).* Such AgNPs stabilized by Tulsi leaf extract were found to have enhanced antimicrobial activity against the well known pathogenic bacterial species. The zone of inhibition was11mm and 10mm were observed on the inoculated plate of *Staphylococcus aureus and E.coli* respectively. This is comparable with present study where the reduced silver nitrate and plant extract showed maximum inhibition of 8±2.3mm against *Staphylococcus aureus* (B11) at a concentration 20µl of 1mM AgNPs.

Govindraju *et al.*, (2010) [26] focused on the biological synthesis of silver nanoparticles using *Solanum torvum* and its antimicrobial activity. *Solnum torvum* mediated silver nanoparticles showed high antimicrobial activity bacteria such as *Pseudomonas aeruginosa* (16.9mm/100µl) and *staphylococcus aureus*(17.6mm/100µl). This is comparable with present study. The reduced silver nitrate and plant extract showed maximum inhibition of 8±2.3mm against *Staphylococcus aureus* (B11) and 10±2.4mm against *Pseudomonas aeroginosa*(D5) at a concentration of 20µl of 1mM AgNPs.

According to the study of Cynthia *et al.*, (2011) [27] the three herbal products from ten selected medicinal plants: *Ziziphus vulgaris, Malvasyl vestris, Onosma bracteatum, Hyssopus officinalis, Ephedra gerardiana, Cordia latifolia, Althaea officinalis, Mentha piperita, Glycyrrhiza glabra, Justica adhatoda*. Antibacterial activity was determined by the agar well diffusion method. Five concentrations (15 mg/ml, 12.5 mg/ml, 10 mg/ml, 7.5mg/ml and 5 mg/ml) were used to check the antibacterial activity found effective against *S. aureus* (2.6 ± 0.1 mm), *P. aeruginosa* (2.33 ± 0.51 mm) and *E. coli* (1.33 ± 0.15 mm) Which is similar to the present Study. Synthesised silver nanoparticle showed maximum inhibition zone against *S. aureus*(8±2.3mm/20µl), *P. Aeruginosa* (10±2.4mm/20µl).

Anarkali *et al., (2012)*[28] studied the biosynthesis of silver nanoparticles (AgNP's) used entire plant extracts (*Mollugo Nudicaulis*) for the reduction of aqueous Ag^+ ions and its increased antimicrobial activity. After the characterization, these nanoparticles are monitored for the antimicrobial activities against clinically isolated organism *M.luteus (*9±6.2nm). This is comparable with present study where the reduced silver nitrate and plant extract showed

maximum inhibition of 7±1.9mm against *M.luteus* (C4) at a concentration 20µl of 1mM AgNPs. This is also correlated with the invention of Veerababu *et al.,* (2012) [29] UV-Visible absorption spectra of the reaction medium containing silver nanoparticles showed maximum absorbance at 455 nm. FTIR analysis confirmed reduction of Ag^{+} ions to Ag^{0} ions in synthesized silver nanoparticles. The SEM and TEM analysis showed the particle size between 5-40nm, and spherical in structure. The silver nanoparticles have shown bactericidal effects (15mm/100µl) against *Micrococcus luteus.*

In present study report the reduced silver nitrate and plant extract showed maximum inhibition of 8.5±1.9mm against *E.faecalis* (B8) at a concentration 20µl of 1mM AgNPs. This is supported by John De Britto *et al.,* 2014 [30]. The synthesized silver nanoparticles were generally found to be effective as antimicrobial agent against the important human pathogen *E.faecalis* (12mm/50µl).

Sangar, (2013) [31] found the antimicrobial activity of *Adhatoda vasica* against *Proteus vulgaris, Pseudomonas aeruginosa, Escherichia coli and Staphylococcus aureus* by Agar disc diffusion method. Their activity was measured at different concentrations of 100 µg, 200 µg, 300 µg, 400 µg, and 500 µg. The crude extracts obtained from the leaf exhibited the activity against *Proteus vulgaris (0.7*mm, 0.6mm, 1mm, 1mm, 2mm), *Pseudomonas aeruginosa* (1mm, 1mm, 1mm, 2mm, 1mm), *Escherichia coli*(1.8mm, 2mm, 0.5mm, 1mm, 2mm) *and Staphylococcus aureus* (2mm, 1mm, 2mm, 2mm, 3mm) respectively.

Where as in the current study the antimicrobial activity of the silver nitrate reduce solution and leaf extract analyzed shows that more inhibitory activity for *Staphylococcus aureus* A4 (pus) 7±1.3mm/20µl, *Pseudomonas aeruginosa* A7 (pus) 10±1.5mm/20µl, *Enterococcus faecalis* B8 (urine) 8.5±1.9mm/20µl, *Staphylococcus aureus* B11 (urine*) 8±2.3mm/20µl, Micrococcus luteus* C4 (throat) 7±1.9mm/20µl, *Pseudomonas aeruginosa (D5) 10±2.4mm/20µl, Enterococcus faecalis (D7)7±0.4mm/20µl* respectively.

CONCLUSION

In the present investigation it is demonstrated that the potential of *J.adhatoda leaf* extract in reducing aqueous to silver nitrate and the formation of eco friendly silver nanoparticles with fairly well defined dimensions. The present study provides evidence's that the leaves are good sources for synthesizing stable silver nanoparticles in lesser time. So fresh leaves could be used as source which economic and easily available for synthesis can be advantages over

other biological process by eliminating the elaborated process. It can also be suitably scaled up for large scale synthesis of nanoparticles. This production through plant, however, will give positive message that nanoparticles synthesized through green routes are much safer for human use.

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