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Research Article

Anti Candidal Biogenic Silver Nanoparticles Synthesised from Leaves of *Azadirhacta indica* and Their Anti Biofilm Effect against Clinical Isolate of *Candida albicans*

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ABSTRACT

Skin infections caused by fungi, such as *Trichophyton* and *Candida* species, have become more common in recent years. This upward trend is concerning,considering the limited number of antifungal drugs available because prophylaxis with antifungals may lead to the emergence of resistant strains and various virulence factors produced by the organism inactivate the drugs. Exploitation of principles of nanotechnology mainly biogenic nanoparticles has the wide variety of potential applications in biomedical, optical, and electronic fields In the present study, silver nanoparticles were synthesized by leaf extract broth of *Azadirhacta indica* **(L.)** and the synthesized nanoparticles were evaluated against biofilm of clinical isolate of *Candida* albicans.Silver nanoparticles were synthesized by the leaf extract broth and synthesized particles were characterized by UV visible spectroscopy,scanning electron microscopy and energy dispersive x ray spectroscopy. Synthesized particles were tested against biofilm of *Candida albicans* under biofilm inhibition spectrophotometric crystal violet assay.Synthesis was primarily confirmed by colour change of the reaction mixture into brown colour. The UV-Vis spectroscopy revealed the formation of silver nanoparticles by yielding the typical silver plasmon absorption maxima at 420 nm and SEM micrograph indicates the uniform spherical particles within the size range of 40-50 nm. The energy dispersive X-ray spectroscopy (EDX) of the nanoparticle confirmed the presence of elemental silver signal as strong peak.Biofilm inhition study revealed that all the tested concentration of silver nanoparticles inhibited biofilm and maximum inhibition was observed in 100µg/ml concentration. Biochemical composition of Biofilm matrix mainly total carbohydrates and total protein was highly reduced in the nanoparticles treatment.

Key Words: *Candida albicans*,biofilm, silver nanoparticles,biofilm matrix

INTRODUCTION

One of the reasons for the growing frequency of hospital acquired *Candida* bloodstream infections is the increasing use of immunosuppresive therapy in cancer and transplant patients, which leads to breakdown of the barrier between the gut and bloodstream [1]. *Candida* cells, like many other microbial organisms, are able to adhere to and colonize surfaces of medical devices, like central venous catheters,voice prostheses, intrauterine devices and prosthetic joints,among others, resulting in the development of a biofilm [2]. Infections due to the presence of fungal biofilms are a major clinical concern as these structured microbial communities, embedded in an extracellular matrix, are characterized by increased resistance to antifungal therapy [3].The current treatment options for fungal biofilm-related infections are very scarce due to the intrinsic increased tolerance of biofilms to antimycotics. In the mid 1990s, *C.albicans* biofilms were found to be resistant to the majority of the antifungal agents [4]. Patients with fungal biofilm infected devices are rarely cured with mono-antifungal therapy and affected devices generally need to be removed [5].

Due to increasing tolerance of the biofilm community to

Fig. 1: Synthesized silver nanoparticles

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Table 1: Effect of silver nanoparticles on biofilm inhibition of *C.albicans*

S.No	Concentration	Biofilm Inhibition (%)	
		Microtitre plate	Nitrocellulose membrane
1	10	41.0	40.4[a]
2	25	56.4	55.0[a]
3	50	69.0	67.1[a]
4	75	79.5	79.0[a]
5	100	96.0	93.4[a]

In column, the mean carries the same letter is statistically significant at (P>0.05) level by DMRT

Table 2. Total carbohydrates and total protein of biofilm matrix of *C.albicans*

S.No	Concentration	Total carbohydrates (mg)		Total protein (mg)	
		Microtitre plate	Nitrocellulose membrane	Microtitre plate	Nitrocellulose membrane
1	10	92.0	80.2	79.2	73.0
2	25	81.0	72.3	66.2	61.4
3	50	74.3	64.3	59.0	53.2
4	75	63.2	61.1	21.0	19.3
5	100	55.1	50.4	10.2	7.0
6	Control	125.0		210.4	

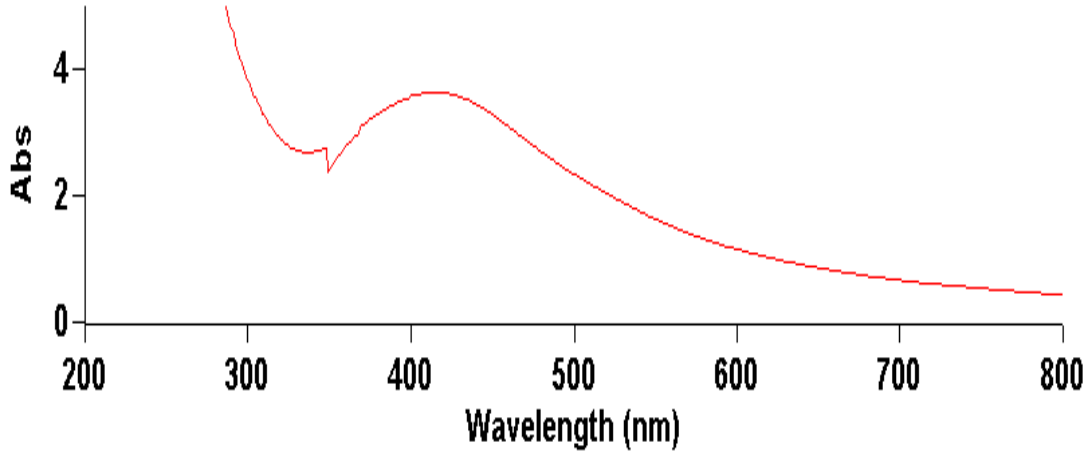

Fig. 2: UV vis spectra of synthesized nanoparticles

antibiotics, biocides and mechanical stress, it has become just as difficult to completely eradicate mature biofilms as it is to completely avoid the presence of planktonic cells, the origin of the biofilm in the water. Common treatments to prevent or remove biofouling include using disinfection, minimizing nutrients in the feed or altering surface materials to prevent bacterial attachment, or clean-in-place (CIP) to remove mature biofilm by chemical or mechanical shear. Several studies have examined the effect of various types of antimicrobial treatment in controlling biofilm formation on medical devices [6, 7, 8]. The vast majority of the chemical agents currently available for biofilm control are broad-spectrum non-specific micro biocide agents [9]. Chloro hexidine, triclosan, and essential oils (*e.g*., Listerine) are the most commonly used and clinically tested antimicrobials [10]. Biofilm-control strategies based on disruption of EPS formation on the surface could be an effective alternative (or adjunctive) approach [11]. In order to control biofilm formation on medical devices and all costs associated, a large number of new strategies and approaches have been developed in the last few years, including: antimicrobial locks (in the case of catheters) [12]; surface modification of biomaterials with antimicrobial coatings [13]; the use of quorum sensing (QS) inhibitors [14], antimicrobial peptides as a new class of antibiotics [15]; enzymes that dissolve biofilms [16], nitric oxide [17], electrical [18] or ultrasound [19] enhancement of antimicrobial activity, or even the application of light activated antimicrobial agents [20]. Nevertheless, nanoscale materials have recently appeared as one of the most promising strategies to control biofilm infections related to indwelling medical devices, especially due to their high surface area to volume ratio and unique chemical and physical properties [21]. A nanomaterial has a diameter ranging from1 and 100 nm, and they can be made from different materials, like copper, zinc, titanium, magnesium, gold, alginate and silver. The use of silver nanoparticles (NPs) is now considered as one of the most promising strategies to combat biofilm infections related to indwelling medical

devices [22]. In the present study,anti biofilm effect of biogenic silver nanoparticles synthesized from leaf extract broth of neem (*Azadirhacta indica*) against biofilm of clinical isolate of *Candida albicans* has been studied.

MATERIALS AND METHODS

Plant materials: Healthy and fresh leaves of *Azadirhacta indica* was collected from the plant grown in home garden. Collected materials were washed in tap water followed by successive washing in distilled water. Washed materials were shade dried. Dried material was homogenized in domestic mixture into fine powder, stored in plastic container at room temperature used for further studies.

Synthesis of biogenic silver nanoparticles: Synthesis of nanoparticles was carried out by leaf extract broth and the broth was prepared by dissolving one gram of homogenized material in 100ml deionised water, filtered through crude filter paper, collected. 50ml of collected filtrate was transferred to 100 ml of beaker and 100ml of 1mM silver nitrate was added and the preparation was kept under magnetic stirrer. Conversion of reaction mixture from pale green to dark brown indicates synthesis of silver nanoparticle Synthesized particles were purified by successive centrifugation at 10,000rpm for 10 minutes and the supernatant was discarded ,the collected pellets were lyophilized and used for further studies.

Characterization: Determination of plasmon absorption maxima of the reaction mixture with UV vis spectra is

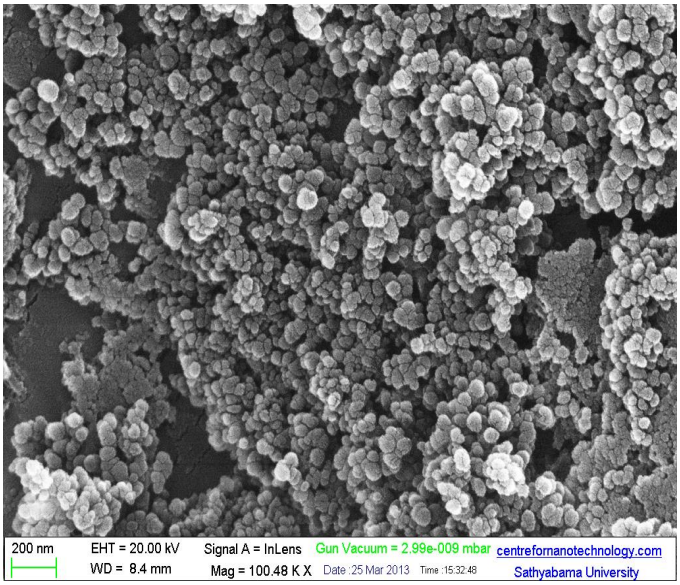

Fig 3: Scanning electron microgroscopy image of silver nanoparticles

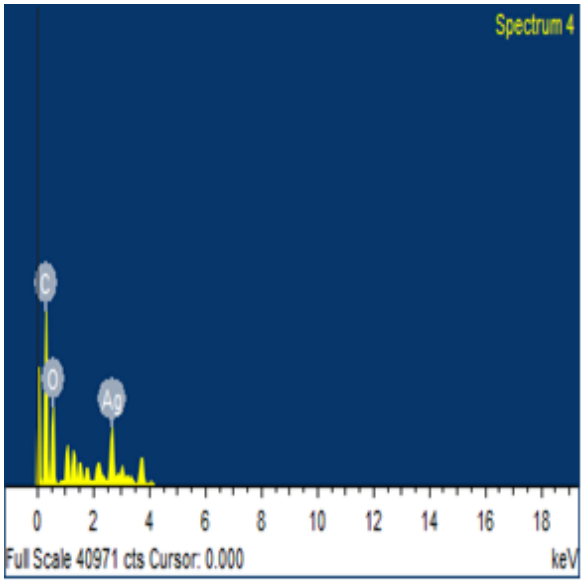

Fig. 4: EDX image of synthesized nanoparticles

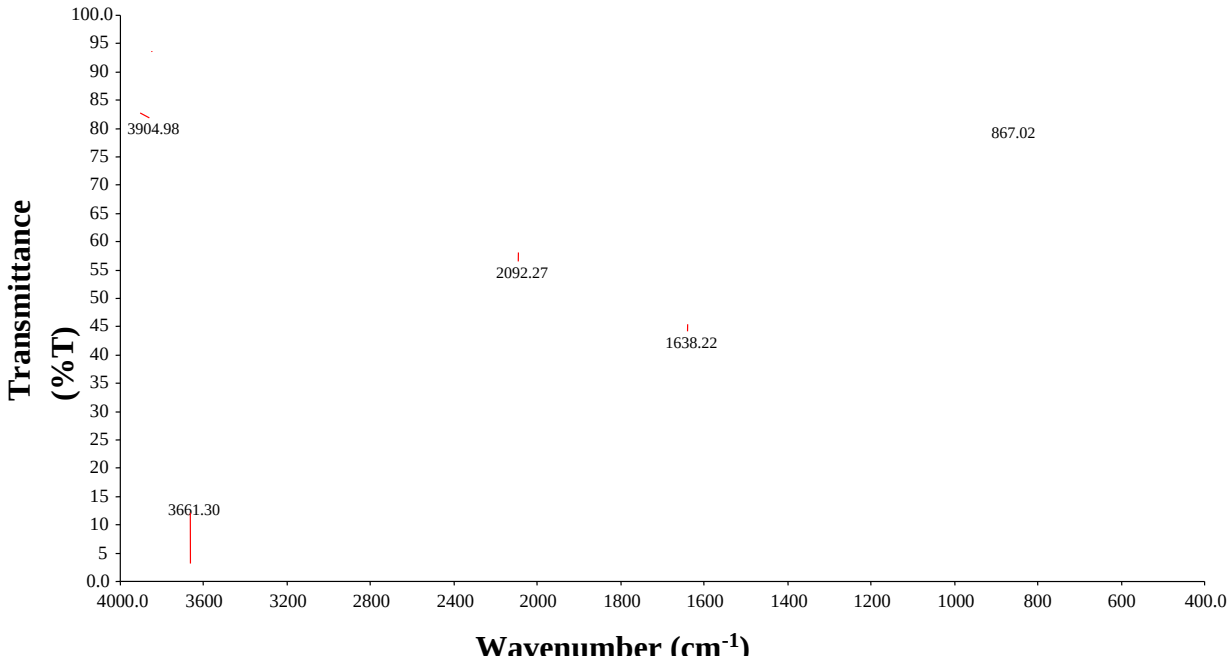

Fig. 5: FTIR spectra of synthesized nanoparticles

(a) Control

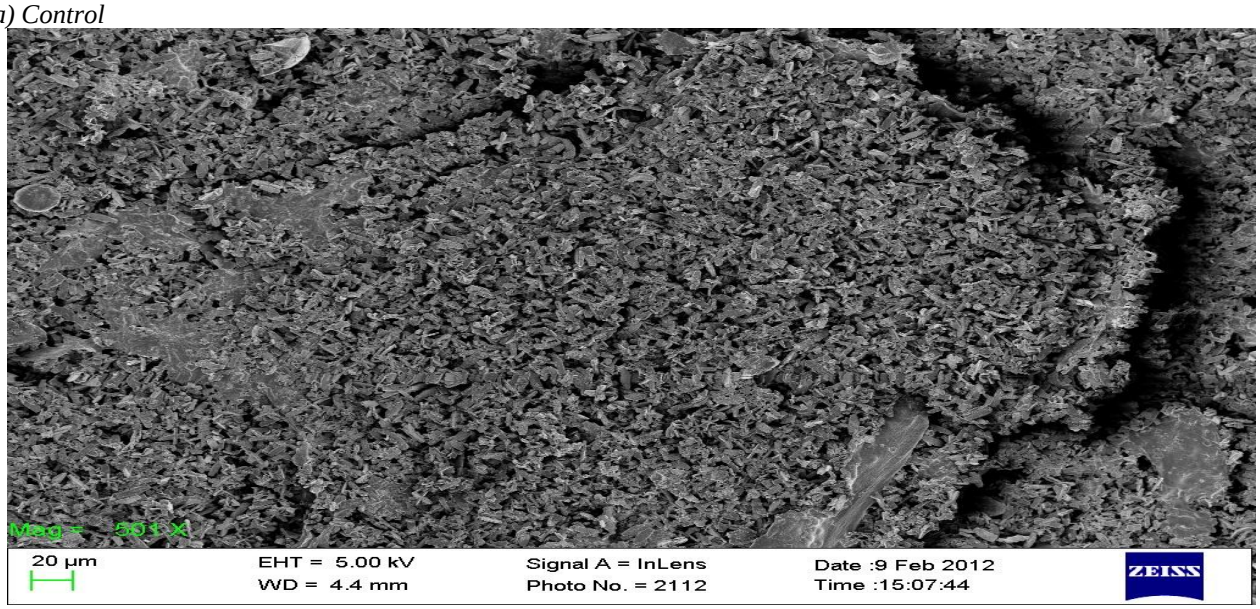

Fig. 6: SEM image of biofilm derived from nitrocellulose membrane C.albicans

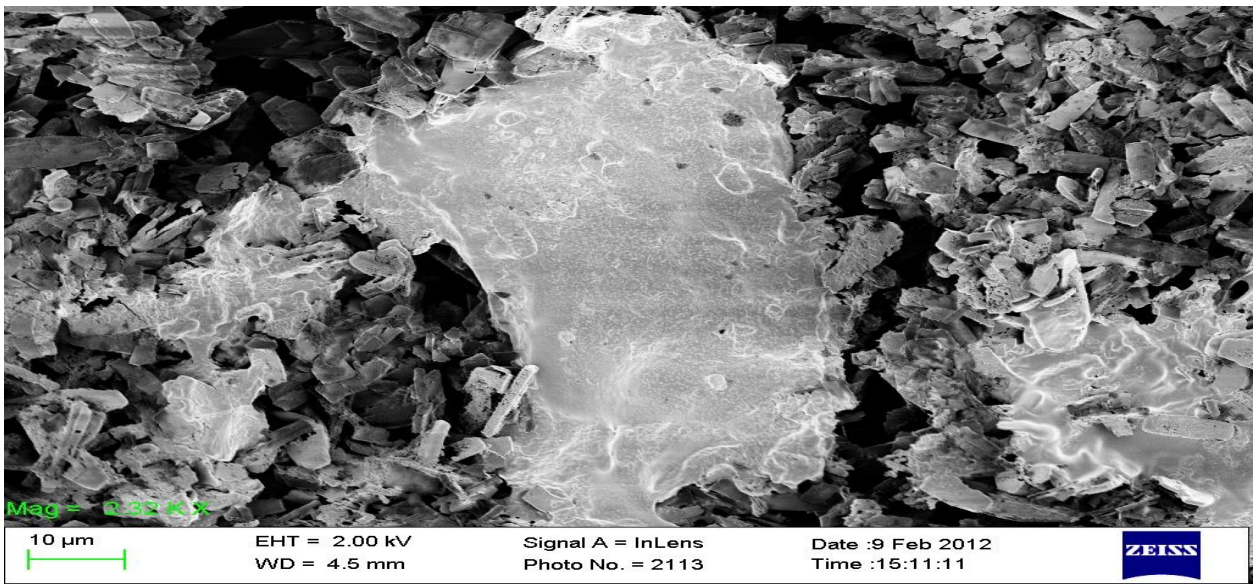

Fig. 6: b.Nanoparticles treated C.albicans

the primary confirmation of the synthesis of nanoparticles. UV vis absorption spectra was carried out with Thermoscientific spectrasacn UV 2700 spectrophotometer operating in the transmission mode. Scanning electron microscopy (SEM) images were recorded by using Carl zeiss subra (Germany) scanning electron microscope equipped with an energy-dispersive spectrum (EDX) capability.

Biofilm inhibition study

Bacterial strain and growth condition: Clinical isolate of *C.albicans* obtained from Madurai Medical college hospital, Madurai, Tamil Nadu, India. The strain was isolated from patient with severe urinary tract infection and maintained on slope of Yeast extract phosphate dextrose (YEPD) slant was used for inocula preparation of the bacterial strain. Cultures was inoculated from fresh slopes and incubated with shaking at 37 °C for 24 hours. Cells were collected by centrifugation and the collected cell debris washed twice in PBS and suspended to OD_{520} prior to use in biofilm experiments [23]

Biofilm inhibition assay: Freeze dried nanoparticles was dissolved in deionized water at different concentration as 10, 25, 50, 75 and 100 µg /ml in sterile screw cap vials, gently shaken well to obtain complete homogenous mixture and used for biofilm inhibition assay. Biofilm inhibition carried out in 96 wall plates and nitrocellulose membrane adopting modified method of biofilm inhibition spectrophotometric assay below [24]. 100µl of cell suspension of *C.albicans* thus prepared was added into 96 well titre plate and different concentration of nanoparticles 25, 50, 75 and 100 µg /ml was added and incubated 37º C for 3 days. After the incubation, the liquid suspension was removed and 100 µl of 1% w/v aqueous solution of crystal violet was added. Following staining at room temperature for 30 minutes the dye was removed and the wells were washed thoroughly, 95% ethanol was added and incubated for 15 minutes. The

reaction mixture was read spectrophotometrically at 570nm.

Biofilm inhibition assay on nitrocellulose membrane: Sterile nitro cellulose membrane filter (Rankem,New Delhi, India) with 47mm diameter and 0.45µm was selected in the present study [25]. 100µl of cell suspension of *C.albicans* thus prepared and the respective concentration of nanoparticles was added to the membrane filter and the filter was transferred to the 6 well tissue culture plate, kept at 37º C for 3 days Five replications and control maintained. After the incubation period, the inoculated filter was taken and the biofilm inhibition assay was carried out by the modified method of spectrophotometric inhibition assay as described earlier. The filter was stained with 1.0% crystal violet and incubated for 1 hour. After staining the filter was washed thoroughly with 1 % ethanol and the washed solution was collected in sterile srew cap vial and the reaction mixture was read at 570nm.

Scanning electron microscopy (SEM): Biofilms were examined by SEM after processing of samples by a freeze-drying technique [26].Biofilms formed on membrane were fixed with glutaraldehyde (2.5%, v/v, in 0?1 M cacodylate buffer, pH 7?0), washed gently three times in distilled water, and then plunged into a liquid propane/isopentane mixture (2 : 1, v/v) at 2196 µC before freeze-drying under vacuum (1026 torr, 1.361024 Pa). Samples were finally coated with gold and palladium and viewed under a Carl zeiss subra (Germany)scanning electron microscope.

Effect of nanoparticles on the biochemical composition of biofilm matrix

Isolation of biofilm matrix: Biofilm matrix material was isolated from the microtitre plate and nitrocellulose membrane [16].Adherent biofilms were transferred to screw cap bottles containing 10 ml distilled water. The bottles were sonicated for 5 min in an ultrasonic water bath and vortexed vigorously for 1 min to disrupt the biofilms. Cell suspensions were then pooled and centrifuged. The collected supernatant used as source for studying biochemical composition mainly protein by Lowry *et al* and total carbohydrate by Dubois *et al* [27].

RESULT AND DISCUSSION

Silver nanoparticles synthesis was primarily confirmed by colour change of the reaction mixture from pale yellow to brown which clearly indicates the formation of silver nanoparticles (Figure 1). The characteristic brown colour due to the excitation of Plasmon vibrations in the nanoparticles provides a convenient signature of their formation. Synthesized silver nanoparticles characterized by UV-Vis spectroscope which reveals a strong broad surface Plasmon peak located at 420 nm (Figure 2). Particle morphology size and shape with scanning electron microscopy reveals spherical particles with the size of 19-21 nm (Figure 3). In EDX, strong signals from the silver particles were observed (72.44% in mass), while weaker signals from C, O atoms are also recorded which confirmed silver nanoparticles (Figure 4).. FT-IR analysis helps to detect the functional groups, structure of a compound and purity of the sample in a given environment in terms of frequencies of radiation present in the nanoparticles. The results of FT-IR spectroscopy of silver nanoparticles which reveals the intensive peaks at 3904.98 cm-1, 3661.30cm-1, 2092.27 cm-1, 1638.22 cm-1 and 867.02 cm-1 (Figure 5).

Biofilm inhibition study revealed nanoparticles with all the tested concentration inhibited biofilm of *C.albicans*. .Results were represented as inhibition percentage of biofilm development (Table 1).Nanoparticles showed distinct effect on biofilm formation with a dose dependent manner. 100µg/ml recorded maximum anti biofilm effect with 96.0 followed by 79.5, 69.0, 56.4 and 41.0 % inhibition at the respective concentration. Dose dependent variation on anti biofilm effect as in microtitre plate was recorded in nitrocellulose membrane which recorded 93.4, 79.0, 67.1, 55.0 and 40.4% of inhibition.

Scanning electron microscopy of the biofilm derived from nitrocellulose membrane treated with silver nanoparticle at maximum inhibitory level reveals complete degeneration of biofilm with weakened cell masses while control revealed compact tightly packed cell aggregrates.(Figure 6 a,b). Surface topography of the membrane coated with silver nanoparticles which reveals complete dispersion of the nanoparticles on the fibre surface of the membrane and the size, shape of the particles shows uniform spherical particles with the size of 50-60 nm. Biochemical composition of biofilm matrix mainly total carbohydrates and total protein was reduced in all the tested concentration of nanoparticles treatment (Table 2). Both total carbohydrates and total protein derived from the biofilm grown on microtitre plate and nitrocellulose membrane was reduced as dose dependent manner.Maximum reduction of biochemical composition was observed in 100 µg /ml.

The application of nanoscale materials and structures, usually ranging from 1 to 100 nanometers (nm), is an emerging area of nanoscience and nanotechnology. Nanomaterials may provide solutions to technological and environmental challenges in the areas of solar energy conversion, catalysis, medicine, and water treatment [2].This increasing demand must be accompanied by "green" synthesis methods. In the global efforts to reduce generated hazardous waste, "green" chemistry and chemical processes are progressively integrating with modern developments in science and industry. Implementation of these sustainable processes should adopt the 12 fundamental principles of green chemistry [28] which are geared to guide in minimizing the use of unsafe products and maximizing the efficiency of chemical processes. Hence, any synthetic route or chemical process should address these principles by using environmentally benign solvents and non toxic chemicals. Nanomaterials often show unique and considerably changed physical, chemical and biological properties compared to their macro scaled counterparts [3.] Synthesis of noble metal nanoparticles for applications such as catalysis, electronics, optics, environmental, and biotechnology is an area of constant interest [29].The application of such a technology can be used for the inhibition of biofilm

formation on the surgical and medical devices which are of higher threat in the process of treatments. Bacteria are able to grow adhered to almost any surface, forming architecturally complex communities termed biofilms [30]. Microbial biofilms develop when microorganisms irreversibly adhere to a submerged surface and produce extracellular polymers that facilitate adhesion and provide a structural matrix. This surface may be inert, nonliving material or living tissue. Biofilm-associated microorganisms behave differently from freely suspended organisms with respect to growth rates and ability to resist antimicrobial treatments and therefore pose a public health problem [31].Metal nanotechnology chemistry can prevent the formation of life-threatening biofilms The present study is undertaken to evaluate the toxic effect of silver nanoparticles synthesized from leaf extract broth of neem (*Azadirhacta indica*) against biofilm of *C.albicans*.Biogenesis was confirmed by colour change of the reaction mixture,plasmon absorption maxima at 420nm, spherical particles with the size range of 19-21nm and FTIR analysis.Biofilm inhibition study revealed all the tested concentration inhibited biofilm and the maximum inhibition was recorded in high concentration. Biofilm inhibitory effect of metallic nanoparticles against pathogenic bacteria has been recently reported which clearly revealed distinct anti biofilm activity of metallic nanoparticles against pathogenic microorganism [32]. Biochemical composition of biofilm matrix was highly reduced in nanoparticles treatment. Maximum reduction of biochemical composition was observed in 100 µg /ml. The matrix is one of the most distinctive features of a microbial biofilm. It forms a three dimensional, gel- like, highly hydrated and locally charged environment in which the microorganisms are largely immobilized [33]. Matrix-enclosed micro colonies, sometimes described as stacks or towers, are separated by water channels which provide a mechanism for nutrient circulation within the biofilm the composition of the matrix varies according to the nature of the organism and reduction of the biochemical composition of the biofilm matrix leads to weakening of the biofilm thus facilitate entry of the drugs [34].

CONCLUSION

Particular attention is oriented nowadays towards the need for antimicrobial textiles and polymers that are able to reduce or eliminate infections completely especially those caused by antibiotic-resistant bacterial strains. Therefore, the developments of nanoparticles with antimicrobial properties have recently received growing interest from both academic and industrial sectors. The present study demonstrated that metallic nanoparticles synthesized by biological method can be used in to prevent or to minimize bacterial infections and will lead to new generation of development of antimicrobial agents to prevent pathogens infection. Further studies on molecular mechanism of biofilm inhibition against pathogenic microorganism will be useful in the development of effective anti microbial agents.

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