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Biosynthesis of silver nanoparticles from *Aspergillus niger* and evaluation of its wound healing activity in experimental rat model.

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Abstract: Among fungi *Aspergillus sp.* are the cost effective source of biomaterial for biosynthesis of silver nanoparticle. In this study, silver nanoparticles were synthesized extracellularly using *Aspergillus niger* and evaluated for its wound healing activity. Two models i.e. excision wound model and thermal wound model were used for this purpose. Its antimicrobial activity was also evaluated by disc diffusion method. In dose at 20mg of silver nanoparticle showed significant reduction in excision wound within 8 days. The results suggested that the silver nanoparticles synthesized from *Aspergillus niger* possess effective wound healing activity when compared with silver nitrate.
Key Words: Silver nanoparticle; Wound healing; Aspergillus; antimicrobial.

Introduction

Silver is one of the most powerful antiseptic materials available naturally and posses low toxicity towards mammalian tissue. Some microbes such as bacteria[1,2,3], yeast[4], fungi[5,6]and algae[7,8] are able to adsorb and accumulate metal and can be used in the reduction of environmental pollution and also for the recovery of metals from waste. This approach had been used for bioformulation of nanoparticles[9]obtained silver particles with an average size of 46Æ9 nm from the bacterium *Bacillus megaterium*. These biosynthetic methods are investigated as an alternative to chemical and physical ones.

Fungi possess some advantages over bacteria; most filamentous fungi have a high tolerance towards metals with high wall-binding capacity and intracellular metal uptake capability[10, 11]. In addition, they are easy to culture on a large scale, especially by the thin solid fermentation method, thus making it possible to easily obtain enough biomass for processing. Another advantage is that the fungus could grow on the surface of an inorganic vector during culture, which could distribute silver nanoparticles in a more efficient way as a catalyst. These advantages, taken together with the results of Pighi et al.led us to examine the potential usefulness of *Apergillus niger*[12] .In our present research we investigated biosynthesis of silver nanoparticle extracellularly and evaluated its wound healing activity.

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Materials and Methods

Materials

Aspergillus niger (NCIM No.617) was obtained from National collection of industrial microorganism, Pune, India. Culture media required for routine cultivation and maintenance of *Aspergillus niger* were procured from Himedia. All chemicals used were of analytical grade and obtained from Fischer Chemicals Ltd, India.

Instruments used were cooling centrifuge (Remi-C-30 BL),electric water bath (Technico), electronic weighing balance(Shimadzu), BOD Incubator (Technico, Chennai), hot air oven (Technico, Chennai), orbital shaker incubator(Remi), orbital water bath(Technico), laminar air flow bench (Technico,Chennai), UV Spectrophotometer (Shimadzu).

Preparation of fungal culture

Aspergillus niger was grown in yeast malt broth at 37°C for 5 days. The flasks were incubated in the shaker incubator at 200 RPM. After 5 days of incubation, the mycelium was separated and washed thrice with deionized water. 20 g of biomass was treated with 200 ml

of deionized water for 72 h at 250C in an Erlenmeyer flask and agitated in the same condition as described earlier. After the incubation, the cell filtrate was obtained by filtration through Watmann filter paper no.1[13].

Synthesis of Silver Nanoparticles

Silver nitrate at 1mM concentration was mixed with 50 ml of cell filtrate in a 250 ml Erlenmeyer flask and agitated at 25ºC in dark along with control[13].

UV–visible spectral analysis

Sample of 1ml was withdrawn from silver nitrate solution incubated with *Aspergillus niger* at different time intervals. The absorbance was measured at a resolution of 1 nm from 200-800nm using UV–visible spectrophotometer [13].

Infra-Red Studies

Infra-Red Spectroscopy was used to investigate and predict any physiochemical interactions between different components in a formulation using FT-IR. The spectra obtained through those samples were compared and interpreted for the shifting of functional peaks.

Particle Size Measurement

Particle sizing experiments were carried out by means of laser diffractometry, using Mastersizer 2000 instrument (Malvern), equipped with HydroMu dispersing unit (Malvern). Measurements were taken in the range between 0.1 and 1000 μm[14].

Zeta potential measurement

Measurements were carried out using a Zetasizer Nano ZS (Malvern) and a titrator MPT-2. An aqueous suspension of silver nanoparticles was filtered through a 0.45 μm PTFE membrane before measurement. The zeta potential was calculated using Henry's equation[14].

Antimicrobial Activity by disc diffusion method

Muller Hinton Agar plates were prepared, sterilized and allow to solidify. After solidification plates were inoculated with bacterial cultures (*Staphylococcus aureus* ATCC.29737, *Bacillus subtilis* ATCC.6633, *Escherichia coli* ATCC.10536, and *Pseudomonas aeruginosa* NCIM. 2945). The sterile disc were dipped in silver nanoparticle solution and placed in seeded agar plates. The plates were incubated at 370C for 24 h and zone of inhibition was measured. The results were compared with standard discs of ofloxacin.

Preparation of Formulation

Preparation of silver nanoparticle ointment

Different concentration of silver nanoparticle (5, 10, 15 and 20mg) were mixed and homogenized with 5 gm of ointment base and used for further study[15].

Stability of formulation

Stability of ointment formulations prepared was evaluated in terms of the changes in physical and chemical parameters, which were likely to affect the stability and acceptability of the formulations[15].

In Vivo Wound Healing Activity

Experimental Animals

Swiss albino rat of either sex, weighing 120–150 g were used for *in vivo* studies and obtained from KMCH College of Pharmacy, Coimbatore, India. The experiments were conducted in accordance with the internationally accepted principles for laboratory animal use and the experimental protocols were approved by the Institutional Animal Ethical Committee (IAEC), KMCRET, Coimbatore, India. The animals were housed in polypropylene cages in standard environmental conditions (20–25 °C), fed with standard rodent diet and water *ad libitum*.

Excision Wound Model

Animals were anaesthetized by open mask method with anesthetic ether. The rats were depilated on the back and a predetermined area of 500 mm2 full thickness skin was excised in the dorsal inter -scapular region Group I served as control (untreated), Group II received standard ointment (neomycin), Group III, IV, V and VI received silver nanoparticle at the concentration of 5, 10, 15 and 20% respectively. The wounds were covered with the appropriate dressings. The dressings were changed daily. During change of the dressings, wounds were inspected and photographed. The progressive changes in wound area were monitored planimetrically by tracing the wound margin on a graph paper every alternate day. The change in healing of wound, i.e. the measurement of wound area on graph paper was expressed as unit (mm2). Wound contraction was expressed as percentage reduction of original wound size.

Wound contraction % =

$$\frac{Wound\ area\ day\ 0 - Wound\ area\ day\ n}{Wound\ area\ day\ 0} \times 100$$

Falling of scab, leaving no raw wound behind, was taken as the end point of complete epithelialization and the days required for this were taken as period of epithelialization.

Thermal Wound Model

Animals were anaesthetised by open mask method with anaesthetic ether and their backs were shaved with electric clippers. They were placed in a mold in such a way that approximately 10% of their body surface area remained exposed. The exposed skin surface of each rat was immersed in 90°C water for 6 seconds, resulting in a grade II burned wound of 10% total body surface area (TBSA). To prevent shock, the animals were resuscitated with intraperitoneal injection of 3–5 ml saline solution. The wounds were covered with the appropriate dressings and treatment as above model and assessed for wound contraction and period of epithelialization. Silver sulphadiazine (1%) served as standard drug.

Statistical analysis

The data are presented as the mean ± SEM. Wound healing was assessed by students unpaired t test. One-way repeated measures analysis of variance (ANOVA) followed by Dunnett's test was used for the statistical evaluation of all other activities**.**

Results and Discussion

Silver nanoparticles had been utilized in various aspects like spectrally selective coating for solar energy absorption, optical receptors, polarizing filters, catalysts in chemical reaction, biolabelling and antimicrobial agents[16]. Application of silver nanoparticles in these fields is dependent on the ability to synthesize particles with different chemical composition, shape, size and mono-dispersity. Development of simple and eco-friendly method would help in developing further interest in the synthesis and application of metallic nanoparticles. In this respect, nature has provided exciting possibilities of utilizing biological systems for this purpose. This comes from the fact that microorganisms while interacting with metal ions have shown to reduce the ions into metallic particles, among microorganisms fungi would be advantageous[17].

It was observed that upon addition of the silver ion (1 mM) into the flask containing the cell filtrate, the color of the medium changed to brown, which indicates the formation of colloidal silver particles in the medium. The brown color of the medium could be due to the excitation of surface plasmon vibrations, typical of the silver nanoparticles[18].

UV Spectral Analysis

The UV-Visible spectra recorded for sample from reaction vessels at different times of reaction.The strong surface plasmon resonance centered at ca. 415–420 nm clearly increases in intensity with time. The solution was extremely stable, with no evidence of flocculation of the particles even several weeks after reaction.

In this study, the UV–Visible spectrum study provides some clues regarding the mechanism of synthesis of silver nanoparticles. There were two absorbance peaks found in the UV range corresponding to 220 and 280 nm. While the peak at 220 nm may be due to absorption by amide bond, the other peak at 280 nm may be attributed to the tryptophan and tyrosine residues present in the protein. This indicates secretion of some protein components into the medium by the fungal biomass which might play important role in the reduction of the metal ions in the form of nanoparticles. Consequently the proteins also may bind to the nanoparticles and enhance the stability.

FTIR Spectral Analysis

FTIR spectrum of silver nanoparticles synthesized by *A. niger*, after 72 h was shown in figure 1. The bands seen at 3453cm−1 and 2040 cm−1 correspond to the stretching vibrations of primary and secondary amines, respectively; while their corresponding bending vibrations were seen at 1637 cm−1. The two bands observed at 1384 cm−1 and 1118 cm−1 can be assigned to the C–N stretching vibrations of aromatic and aliphatic amines, respectively.

FTIR spectrum also proved the same. It showed bands at 3453 cm−1 and 2040 cm−1 were assigned to the stretching vibrations of primary and secondary amines respectively; while their corresponding bending vibrations were seen at 1673cm−1. The two bands observed at 1384cm−1 and 1118 cm−1 can be assigned to the C–N stretching vibrations of aromatic and aliphatic amines, respectively (Figure 1). The overall observation confirms the presence of protein in the samples of silver nanoparticles. It is reported earlier that proteins can bind to nanoparticles either through free amine groups or cysteine residues in the proteins[3,19].

Particle Size Determination

Particle size determination of the formulated nanoparticles was shown under different categories like size distribution by volume, by intensity. Laser diffraction revealed that particles obtained are polydisperse mixture with the size ranging between hundreds on nanometers and micrometers (Figure 2). The average diameter of the particles was found to be 820 nm and the peak intensity was found to be 6.97%. The size distribution by volume gives a bell shaped pattern which indicates the wide range size distribution of nanoparticles in the sample formulation. The volume% of the sample was found to be in the range of 100-1000d.nm.

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Zeta Potential Determination

The zeta potential of the sample was determined in water as dispersant. The zeta potential was found to be -13.7. The negative value confirms the repulsion among the particles and there by increase in stability of the formulation (Figure 3).

The zeta potential was found to be -13.7. The negative value which confirms the high negative value will lead to repulsion among the particles and there by increasing the stability of the formulation significantly.

Antimicrobial activity

Silver nanoparticle produced by *Aspergillus niger* shows antimicrobial activity in 50µl concentration against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli,* and *Pseudomonas aeruginosa*. The results are shown in the table1. The maximum zone of inhibition was observed with *Staphylococcus aureus* which was about 15mm in diameter. Whereas the cultures of *Bacillus subtilis*, *Escherichia coli*, *and Pseudomonas aeruginosa* were also show zones of inhibition which was about 11mm, 10mm, 14mm in diameter respectively.

The antibacterial activity of freeze-dried silver nanoparticle bacterial *Escherichia coli, Bacillus subtilis, Pseudomonas aeruginosa* and *Staphylococcus aureus* was evaluated by the disc diffusion method. It was found that the freeze-dried silver nanoparticle showed better inhibitory zone when compare to control. This clearly demonstrates that the antimicrobial activity increases due to silver nanoparticles synthesized from *Aspergillus niger*

than the silver nitrate. The antimicrobial activity of silver nano particles was coincides with reports of Thawatchai et al. [20]

Stability of the Formulations

There was no evidence of phase separation, development of objectionable odour or any other evidence of physical instability and effect on storage at varying temperature.

Pharmacological Evaluation of Wound Healing Activity

Excision wound model

Wound contraction is a factor, which indicates rate of reduction of unhealed area during the course of treatment. Greater the reduction better is the efficacy of medication. It may be seen that the fastest healing of wound took place in case of animals received different concentrations of silver nanoparticle; complete healing was obtained within 8 days. The least rate of wound healing was seen in control group, which received no treatment. Wound failed to heal even after 8th day although the unhealed area was smaller than the control for neomycin ointment. The rate of wound contraction in the silver nano-treated group was significantly (p<0.05) less than that in control animals (Table 2).

The evaluation of period of epithelialization revealed that groups received Silver nanoparticle required a total period of about 9 days for complete epithelialization and healing, whereas the control and standard groups took about 12 and 10 days, respectively (Figure 4).

In the excision wound the animal treated with silver nanoparticles showed significant reduction in wound when compared with control. The complete healing obtained within 8 days in silver nanoparticle treated animals where as in standard and control are 10 and 12 days respectively. In the burn wound the re-epithelialization obtained within 14 days in silver nanoparticles treated animals where as control group took 18 days for re-epithelialization.

Thermal Wound Model

The progress of the wound healing induced by silver nanoparticles ointments (5%, 10%, 15%, 20%w/w) treated groups, (control) untreated group and silver sulphadiazine (standard drug) treated group of animals are shown in Table 3. It is observed that the wound contracting ability of the silver nanoparticle ointment in different concentrations was significantly greater than that of the control (i.e. untreated group). The 20%(w/w) silver nanoparticle ointment treated groups showed significant wound healing from the fourth day onwards, which was comparable to that of the standard drug treated group of animals. Ten percent silver nanoparticle ointment treated group of animals showed significant wound contraction from the eighth day onwards and achieved 100% with the wound closure time of 13 days.

It was found that the animal group treated with the silver nanoparticles ointments show significant reduction in the period of epithelialization. The control group of animals took 18 days for re-epithelialization where as the silver nanoparticle group took only about 13 days. The standard group also took 15 days for the complete epithelialization (Figure 5).

Conclusion

For many years, silver sulfadiazine has been the standard treatment for burns, but some of the benefits of pure silver appear to be lost. Recent advances in nanotechnology have resulted in the ability to produce pure silver as nanoparticles. Our findings not only confirm the efficient antimicrobial property of silver nanoparticles, but also implicate the ability of silver to modulate the cytokines involved in wound healing.

Table 1 Anti microbial activity of silver nanoparticle by disc diffusion method

Microorganisms	Zone of inhibition(mm)		
	Silver nanoparticles 50µg/ml	Silver nitrate solution	Standard antibiotic disc
Stapylococcus aureus	15	10	19
Bacillus subtilis	11	15	20
E.coli	10	7	19
Pseudomonus aeuroginosa	14	12	20

Table 2 Percentage of wound contraction in excision wound model

Groups	Percentage of wound contraction		
	Day 4	Day 6	Day 8
Control	48.12±0.13	71.68±0.27	71.47±1.003
Neomycin1%	49.78±0.15***	76.88±0.19**	79.34±0.34ns
SNP5%	50.98±0.36**	75.20±0.26***	98.22±0.77**
SNP10%	56.63±0.33***	82.67±0.27***	97.37±0.79***
SNP15%	58.67±0.12**	84.52±0.16***	98.15±0.30***
SNP20%	59.37±0.18***	83.58±0.17***	98.98±0.56***

*p<0.05, **p<0.01, ***p<0.001, ns – non significant n=6; values are mean ± SEM Statistical analysis of data was carried out by one way ANOVA followed by Dunnett's test.

Table 3 Percentage of wound contraction in thermal wound model

Treatment (mg/gm)	**Percentage of wound contraction**				
	Day 4	**Day 6**	**Day8**	**Day 10**	**Day 12**
control	16.67±0.13	24.55±0.17	34.60±0.46	57.17±0.08	85.92±0.10
Sulphadiazine1%	35.43±0.18	48.00±0.10**	77.93±0.11**	86.35±0.15**	97.5±0.16**
SNP5%	47.13±0.12**	75.38±0.11**	85.42±0.09**	95.15±0.21**	98.82±0.11**
SNP10%	48.13±0.11**	76.18±0.17**	86.13±0.12	97.60±0.17	98.90±0.13
SNP15%	49.13±0.24**	77.42±0.13**	86.40±0.17**	98.15±0.12**	98.82±0.12**
SNP(20%)	50.82±0.16**	78.33±0.14**	87.77±0.14***	98.28±0.42***	99.680.±0.16***

*p<0.05, **p<0.01, ***p<0.001, ns-non significant n=6; values are mean ± SEM Statistical analysis of data was carried out by one way ANOVA followed by Dunnett's test.

Figure 1 FT-IR spectrum of biosynthesized silver nanoparticles

Figure 2 Particle size distribution curve for silver nanoparticles

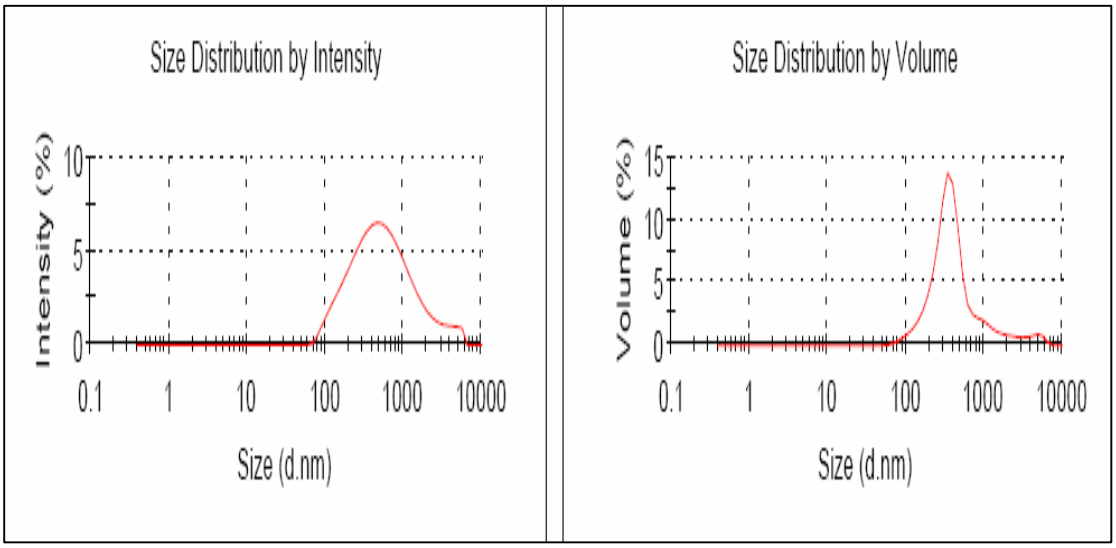


Figure 3 Zeta Potential of silver nanoparticle

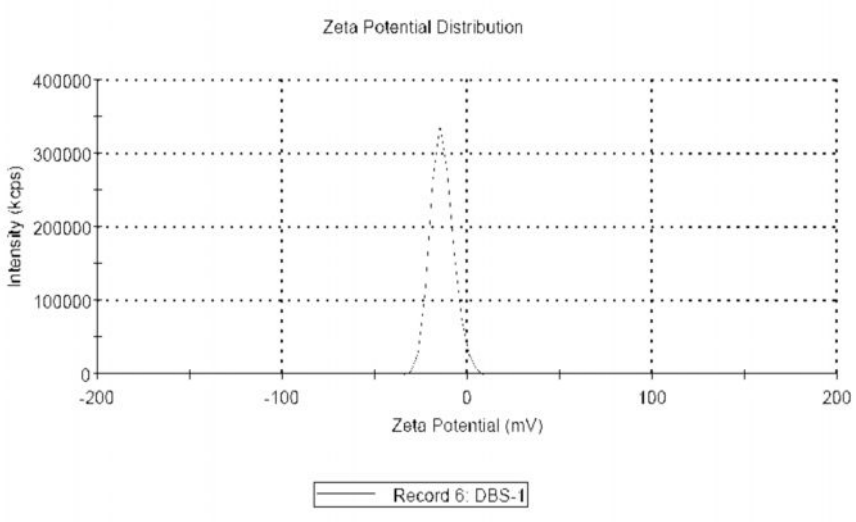
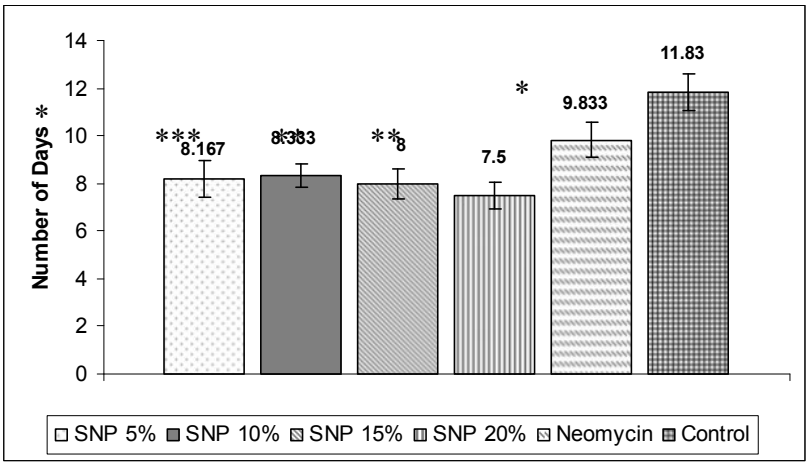
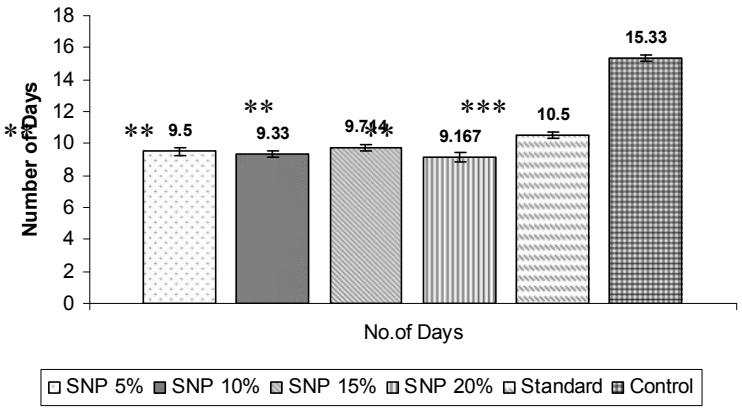


Figure 4 Graph showing period of epithelialization in excision wound model



***p<0.05, **p<0.01, ***p<0.001, ns – non significant**
n=6; values are mean ± SEM .Statistical analysis of data was carried out by one way ANOVA followed by Dennett's test.

Figure 5 Graph showing period of epithelialization in thermal wound model



*****p<0.001, **p<0.01 n=6; values are mean ± SEM. Statistical analysis of data was carried out by one way ANOVA followed by Dunnett's test.**

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