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Wound healing and angiogenesis of silver nanoparticle from *Azadirachta indica* in diabetes induced mice

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Abstract
Nanomaterials are potential to treat the human diseases HIV, malaria, TB, diabetes and cardiac problems. The present work was aimed to detect the wound healing and angiogenesis of silver nanoparticles (SNP) extracted from *Azadirachta indica* (*A. indica*). 20g of *A. indica* Leaf contains 20mg of SNP. SEM and XRD analysis showed the size and characterization of the silver particle was less than 100nm. Thermal wound induced in diabetes animals model was used for wound healing effects of SNP. The wounds decreased in size gradually with time, closed at 2 weeks in normal mice and at 3 weeks in diabetic mice. In diabetic mice at 15 days, a significant difference in (P<0.01) wound closure was noted on the skin. Histopathological observation of skin showed improvement in the surface epithelium. The results of the present study revealed that the SNP from *A. indica* considered as a potential drug target for tissue injury.

Keywords: Silver nano particles, diabetes mellitus, angiogenesis, thermal wound, *Azadirachta indica*

1. Introduction
Nanotechnology is the application of nanoscience, engineering and technology to produce nano materials and devices. Nanomaterials are at the leading edge of the rapidly developing field of nanotechnology of great interest due to their unusual optical, chemical, photoelectron chemical and electronic properties [1]. Nanotechnology has potential to detect meticulously the human diseases such as HIV, malaria, TB, diabetics, cardiac problems and cancer. Drug delivery systems using nanotechnology is a promising aspect. Nanotechnology, unlike any other technology can find applications in many areas of human life. The nanotechnology will play an increasingly crucial role in many key technologies of the new millennium. It is gaining important in areas such as catalysis, optics, biomedical sciences, mechanics, magnetic, and energy science [2].

Bio nanotechnology involves biological production and utilization of nonmaterial using plant and animal as well as microorganisms based products. Bio nanotechnology is of crucial importance for many reasons. The synthesis of nonmaterial over a range of chemical composition and high mono disparity is still challenging in material science. Several manufacturing techniques usually employs atomistic, molecular and particulate processing in a vacuum or in a liquid medium are in use [3]. Most of the techniques are capital intensive, as well as inefficient in materials and energy use. Hence, there is an ever growing need to develop clean, non-toxic, and environmentally benign synthesis procedures. Consequently, researchers in nanoparticle synthesis have turned to biological systems. It is well known that many organisms can provide inorganic materials either intra or extracellular [4].

The development of reliable green process for the synthesis of silver nanoparticles is an important aspect of current nanotechnology research. Nanomaterials such as Ag, Au, Pt and Pb have been synthesized by different methods, including hard template, using bacteria, fungi and plants. Among these, silver nanoparticles play a significant role in the field of biology and medicine due to its attractive physiochemical properties. The towering environmental concerns had triggered the researchers to device novel methods of synthesizing the nanomaterials in biological systems such as bacteria, fungi and plants, termed as “green chemistry” approaches. Biosynthesis of silver nanoparticles using bacteria fungi, yeast, and plants were well documented [5].

Silver micro and nanoparticles are well known disinfectant reported in several reports. Microbes cannot buildup resistance against silver, as they are against conventional and narrow-target antibiotics, because the metal attacks a broad range of targets in the organisms, which means that they would have to develop a host of mutations simultaneously to protect themselves. Gold nanoparticles can be utilized in biosensor devices for the detection of viruses

And bacteria. The progress of development and application of nano colloids in medical science provides an entirely new scope for detecting various ailments [6].

Wound healing is regarded as a complex and multiple step process involving integration of activities of different tissues and cell lineages. The most well documented and commonly used application of silver nanoparticles for this is in the use of wound dressings. The study concluded that silver nanoparticle dressing has a beneficial effect of protecting the wound site from bacterial contamination. Compared with other silver compounds, AgNPs seem also to promote healing and achieve better cosmetics after healing [7].

The medicinal plant *A. indica* provides an answer to many incurable diseases. Traditionally *A. indica* products have been used against a wide variety of diseases which include heat-rash, boils, wounds, jaundice, leprosy, skin disorders, stomach ulcers, chicken pox, etc. Modern research also confirms *A. indica*'s curative powers in case of many diseases and provides indications that *A. indica* might in future be used much more widely [8]. In the present study an attempt was made to identify the wound healing effect of silver nanoparticles of *A. indica* using diabetic induced animal model.

2. Materials and methods

2.1 Collection of plant material

A. indica plant leaves are collected freshly from Mohammad Sathak College of Arts & Science, Sholinganallur, Chennai, Tamil Nadu, and India. The leaf samples were free from pesticides and other contaminants.

2.2 Preparation of plant extract

Aqueous extract of *A. indica* was prepared using freshly collected leaves (20 g). The leaves are thoroughly cleaned with running tap water and cut into small pieces and added 100ml distilled water and boiled in the water bath at 80 °C for 1 hour. This extract was filtered through normal filter paper.

2.3 Synthesis of silver nanoparticle

1mM $AgNO_3$ aqueous solution of silver nitrate was freshly prepared for synthesis of silver nanoparticles. 20ml of Silver nitrate solution was added to 20gm of leaf extract added in a conical flask. The plant extract with the substrate was incubated in dark (to minimize the photo activation of silver nitrate) at 37 °C under static condition. A control set up was also maintained without *A. indica* Leaf extract. The quantity of the synthesized silver nanoparticle was assessed.

2.4 Characterization of silver nano particles

2.4.1 UV-VIS spectra analysis

The reduction of metallic silver ions was monitored by measuring the UV-VIS spectrum after about incubation. A small aliquot was drawn from the reaction mixture and a spectrum was taken on a wave length from 300 nm to 800nm on UV-VIS spectrophotometer.

2.4.2 XRD analysis

For the XRD and SEM analysis, the suspension of nano particles were dried into powder and about 1mg fine powder was used for the analysis. XRD analysis was recorded by using philips PW 1830 X-ray generator operator at a voltage of 40 kev.

2.4.3 SEM analysis

SEM analysis was carried out on JEOL JSM 6360 A (SEM) and using JEOL JSM 1600 A fine coated for uniform.

2.5 *In vivo* studies

2.5.1 Animals used in this study

Twenty week old male Swiss albino mice (Department of Animal house, Mohamed Sathak College of Pharmacy, Chennai, India) weighing between 28 and 32 grams, were used for all thermal injury experiments. Diabetic induced and non-diabetic control male mice were used for the impaired wound healing animal model.

2.5.2 Thermal wound model

For the experimental section, animals are divided into 5 groups each group carries three animals.

Group I: Control

Group II: DM + with wound

Group III: DM + with wound + 2.5mg SNP + 3.2mg SSD ointment

Group IV: DM + with wound + 5mg SNP + 3.2mg SSD ointment

Group V: Thermal wound + 2.5mg SNP + 3.2mg SSD ointment

2.5.3 Induction of diabetes

In this study, 15 male mice are selected for use. The base line blood glucose level of each of the animal was taken (normal control). For diabetes induction streptozotocin (STZ) was used (27mg of STZ in 1.2 ml of Citrate buffer). STZ was freshly dissolved in 0.1M citrate buffer pH 4.5. Single dose of STZ was administered intra peritoneal (IP) injection of 150mg per kg body weight. For the IP injection of STZ, the mouse was held in one hand in dorsal position. The injection site was swabbed using ethanol solution and the designated amount of STZ (0.2ml) was injected in the caudal abdominal cavity using sterile needle. Diabetes develops gradually and may be assessed after 4 days.

2.5.4 Serum glucose levels

Usually a serum glucose level of about 180-500 mg/dl indicates the induction of diabetes mellitus. Severity of the induced diabetic state was assessed by daily monitoring of blood glucose levels. For the determination of blood glucose using glucocheck, the blood glucose levels of these animals were measured at 30, 60 & 120 minutes intervals through tail tipping using a glucometer.

2.5.5 Thermal wound model

The dorsum of each mouse was carefully shaved from the base of the tail to the base of the neck and laid on the burn template following anaesthesia. The syringe was placed horizontally into a water bath at 70 °C for 35seconds, followed by immediately placement of the mouse into on iced water bath to stop the burning process. This model would achieve approximately 10% deep partial thickness thermal injury of total body surface area. The mouse was injected with sterile saline intra peritoneally (1ml) for fluid resuscitation.

The wounds were covered with the appropriate dressings and the animals were bandaged. The dressings were changed daily. During changing of the dressings, wounds were inspected and photographed. Mice were sacrificed at 5, 10, 15, 20 & 25 days after injury, and the day of wound closure for sample collection. Wound healing is defined as the time at which the wound is completely covered by scab. Tissue samples from scalded wound skin (approximately 3X2 sq.cm) of the mice were harvested. Each skin sample was divided for histological analysis.

2.5.6 Animal treatment

2.5.6.1 Preparation of silver nanoparticle ointment

Different concentrations of silver nanoparticle (5, 10 & 15 mg) were mixed and homogenized with 5gm of silver sulfadiazine (SSD) white ointment base and used for the treatment of animals.

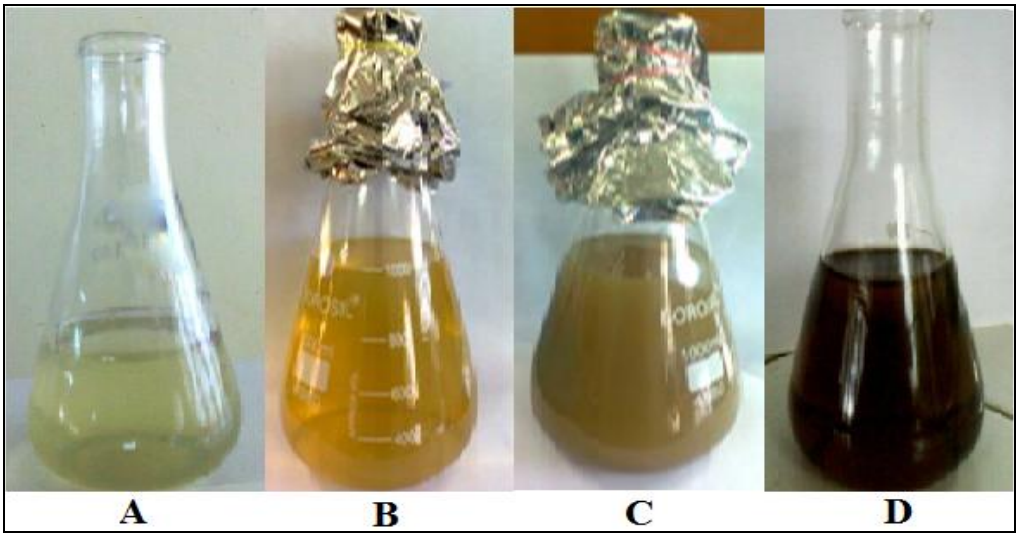
2.5.6.2 Preparation of dressings

Group 3 animals were treated with 2.5mg SNP and 3.2mg SSD coating. Group 4 animals were treated with 5mg of SNP and 3.2mg of SSD coating. Group 5 animals were treated with 2.5mg SNP and 3.2mg SSD.

3. Results and discussion

3.1 Synthesis of Silver nanoparticles

Azadirachta indica was used to produce silver nanoparticles and the reduction of silver ions into silver particles during exposure to the plant extract is followed by colour change from colour less to color, depends on the medicinal plant extract**.** It is known that silver nanoparticles exhibit yellowish brown color in aqueous solution due to excitation of surface plasmon vibrations in silver nanoparticles. As the plant extract was mixed in the aqueous solution of the silver ion complex, it changes the color from watery to yellowish brown due to reduction of silver ion, which may be the indication of formation silver nanoparticles (Plate 1).



A-D: *A. indica* Leaf extract showing the colour change in the synthesis silver nanoparticles on incubation time.

Plate 1: Extraction and Synthesis of silver nanoparticles from *A. indica*

3.2 Quantification and Characterization of silver nanoparticles

3.2.1 UV-VIS Spectra analysis

Synthesized silver nanoparticle were confirmed by UV-VIS spectra at regular time intervals and the absorption maxima was scanned at the wavelength of 300–600nm. The maximum absorption (λ_{max}) spectrum of nanoparticles was observed at 425nm. The plant extract showed to synthesize the silver nano particles by the indication of suitable UV visible spectrum. Similar phenomenon was demonstrated by Chandran *et al*., 2006 [9]. (Figure 1).

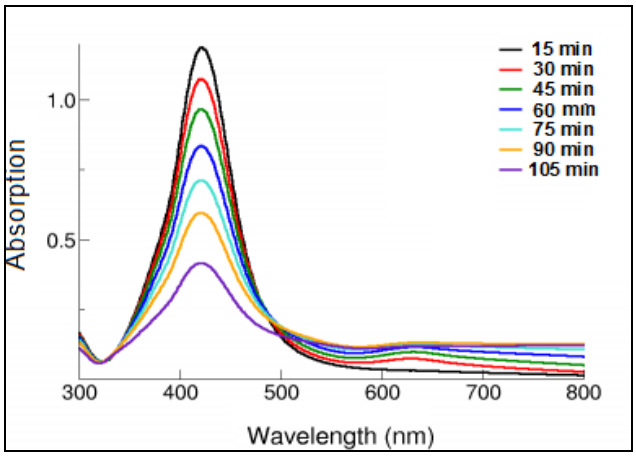


Fig 1: Absorption spectrum of silver nanoparticles of *A. indica*

Absorbance spectrum of *A. indica* Leaf extract treated with silver nitrate solution scanned from 300-800nm in UV-VIS spectrophotometer.

3.2.2 X-Ray Diffraction (XRD) analysis

Structural characterization has been performed using XRD analysis and the typical XRD pattern for sample *A. indica* silver nano particles was shown in Figure 2 and found that four XRD peaks 1, 2, and 3 appear at 28°, 32°, 38°, and 44° due to reflections from different planes of silver. In addition to these three peaks there are unidentified peaks appeared in the XRD pattern. Spectra of pure crystalline silver structures have been published by the Joint committee on powder diffraction standards. A comparison of our XRD pattern with standard confirmed that silver particles formed in our experiments were in the form of nanocrystals, as peak evidenced by the peaks at 2O values of 28, 32, 38 ºC and 44 ºC corresponding to 16, 20, 34 and 12 planes of silver respectively. The similar pattern was obtained by Geethalakshmi and Sarada, 2010 [10].

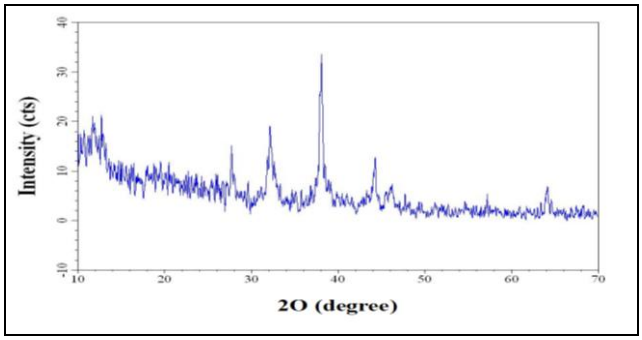
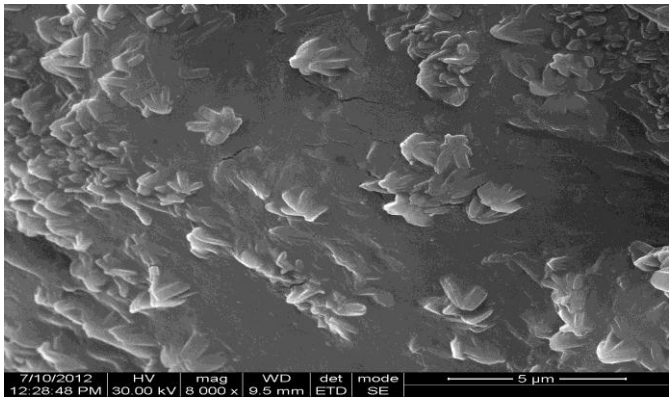


Fig 2: XRD pattern of *A. indica* silver nanoparticles

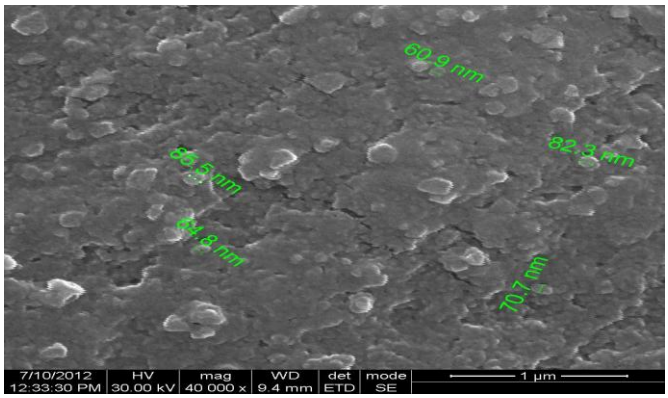
3.2.3. Scanning Electron Microscopy (SEM) Analysis

SEM analysis of the $AgNO_3$ and synthesized silver nanoparticles were clearly distinguishable owing to their size difference. *A. indica* silver nano particles were subjected for the SEM analysis in which the SEM photos clearly shown that in the room temperature synthesized nano particles in the size obtained was less than 100nm and ranges from 60-85nm whereas the shapes were spherical and cubical (Plate 2 and 3). The SEM supported characterization of herbal mediated synthesized silver nano particles were previously done by the Roy and Barik [11].



Cubical structure of SNP form *A. indica* by SEM analysis (8,000X)

Plate 2: SEM Analysis of Silver nanoparticle from *A. indica*



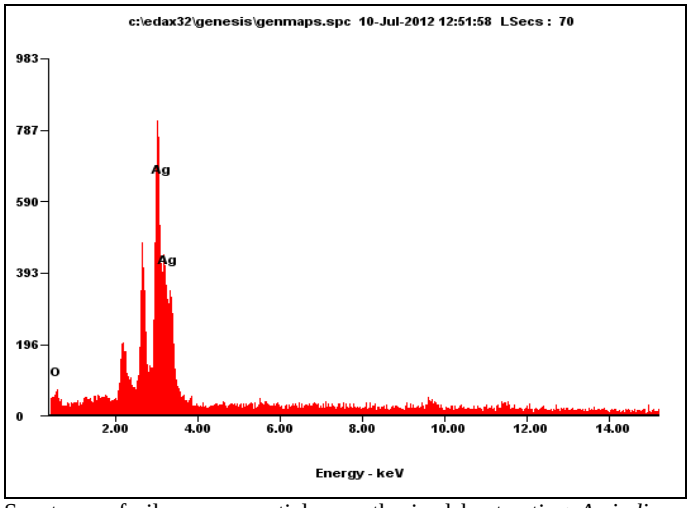
Size of SNP form *A. indica* by SEM analysis (40,000X)

Plate 3: SEM Analysis of Silver nanoparticle from *A. indica*

The SEM image showing the high density silver nanoparticles synthesized by the A. *indica* Leaf extract further confirmed the development of silver nanostructures by the plant extract. The formation of silver nanoparticles as well as their morphological dimensions was done by using SEM analysis demonstrated that the average size was from 60-85nm with inter-particle distance. Similar phenomenon was reported by Chandran and group [12]. The shapes of the silver nanoparticles were proved to be spherical. The nanoparticles were not in direct contact even within the aggregates, indicates the stabilization of the nanoparticles by a capping agent (proteins secreted by plant leaf extracts). The presence of secondary materials capping with the silver nanoparticles may be assigned to bio-organic compounds from leaf extracts [13].

3.2.4. EDAX Measurements

EDAX analysis, the leaves extracts reduced silver nano particles were dried and drop coated on to carbon film and performed on instrument. The significant presence of elemental silver was confirmed from the strong silver peak obtained from the EDAX spectrum as shown in Figure. Metallic silver nanoparticles generally show typical optical absorption peaks approximately at 3kV due do Surface Plasmon Resonance [12]. The EDAX peaks of Ag along with C as the mixed precipitates present in the reaction medium. The EDAX profile showed a strong elemental signal along with oxygen, which may have originated from the biomolecules bound to the surface of the nanoparticles [13]. (Figure 3)



Spectrum of silver nanoparticles synthesized by treating *A. indica* ethyl acetate extract with $AgNO_3$ solution.

Fig 3: EDAX spectrum of silver nanoparticles synthesized from *A. indica*

3.3 *In vivo* wound healing Activity

3.3.1. Induction of DM

The selected animals were induced with the diabetes after 15 days from the date of STZ induction, the blood glucose levels were checked and tabulated (Table 1). The Blood glucose level was significantly increased and reaches >300mg/dl during the period of treatment which confirm the induction of diabetes mellitus. At this pathological condition normally it takes longer time for wound healing.

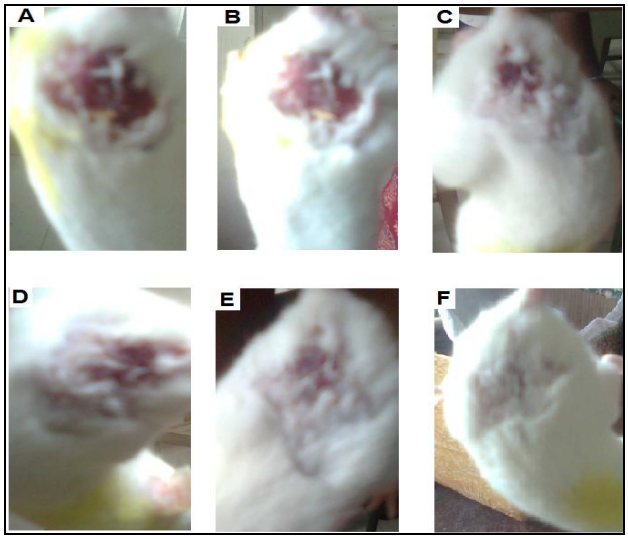
Table 1: Blood Glucose level of Diabetes Mellitus induced mice

Groups	Blood Glucose levels (mg/dl)			
	0th day	5th day	15th day	20th day
Group I	103±9	189±3*	279±14**	320±15***
Group II	105±7	192±2*	271±10**	331±14***
Group III	110±2	200±6*	281±12**	319±12***
Group IV	120±8	198±6*	268±9**	321±10***
Group V	116±1	201±1*	283±11**	301±12***

The values are Mean ± SD (n=3) *** P<0.001, ** P<0.01, * P<0.05– Statistically Significant

3.3.2. Wound healing activity

To compare the difference in wound healing between untreated and treated thermal wound by silver nanoparticles extracted from *A. indica* with and without SSD drug, the measurement of maximum load and tensitle strength of full thickness wound skin from mice at each 5 days interval till the end of treatment. The average percent (%) increase in maximum load is plotted versus day after treatment. Thermal wound were 58% stronger than controls 7 days after wound creation, and 40% stronger than controls at day 10 (Plate 4).



Representative photographs of the mouse with thermal wound splinting model during the treatment with SNP. A-0th day, B-5th day, C-10th day, D-15th day, E-20th day and E-25th day.

Plate 4: Wound healing and Angiogenesis of Silver nanoparticles from *A. indica*

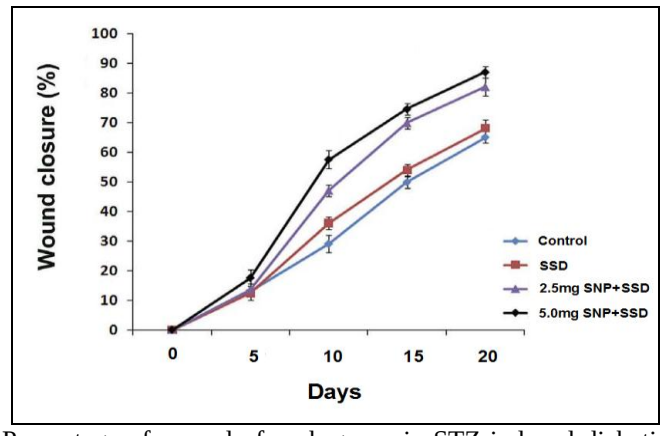
Mice receiving STZ have significant elevation of blood glucose level (>300 mg/dl) after 3 weeks, which was sustained throughout the duration of the study. The wound healing of various treatments was evaluated in a full-thickness wound model. The wounds decreased in size gradually with time, closed at 2 weeks in normal mice and at 3 weeks in diabetic mice. There were unexpected since skin wounds of mice are known to heal efficiently and there is great percentage of improvement. However, in the diabetic rats, periodical observation of animals at 5 days showed a significant increase (P<0.05) in the rate of contraction of wounds in the SNP treated groups when compared to control group. In the diabetic rats at 15 days, a significant (P<0.01) wound closure was noted on the skin. However, all groups attained full closure by the end of the third week.

In Table 2, the average size of the wound on the day of wound creation to till the end of the treatment period was measured and indicated, which shows the maximum level of significant (P<0.01) contraction of the thermal wound was observed over the period of treatment. To investigate the *in vivo* wound healing potential of silver nano particles, we induced diabetes using mouse model with and without creating thermal wounds. The nanoparticles were then transplanted into dermis at a distance of 0.5cm from the wounds. Wound healing results showed that nanoparticle treated wounds displayed accelerated wound healing at days 5, 10, 15, 20 and 25 compared with those treated with drug alone (Figure 4). Because the splints did not completely adhere to the mouse skins as a result of hair growth and movement, wound data after 25 days were not obtained.

Table 2: Percentage of wound contraction in thermal wound model

	Treatment	**Percentage of wound contraction**			
	mg/gm	Day 5	Day 10	Day 15	Day 20
Group I	Control	-	-	-	-
Group II	SSD	35.43±0.18	48.00±0.10	77.93±0.11	86.35±0.15
Group III	SNP+SSD	46.32±0.12**	78.43±0.21**	83.44±0.07**	94.14±0.21**
Group IV	SNP+SSD	47.44±0.21**	75.23±0.17**	84.14±0.22**	97.87±0.17**
Group V	SNP15%	49.74±0.24**	76.44±0.13**	85.03±0.17**	98.05±0.12**

The values are Mean ± SD (n=3)
*** P<0.01 – Statistically Significant*

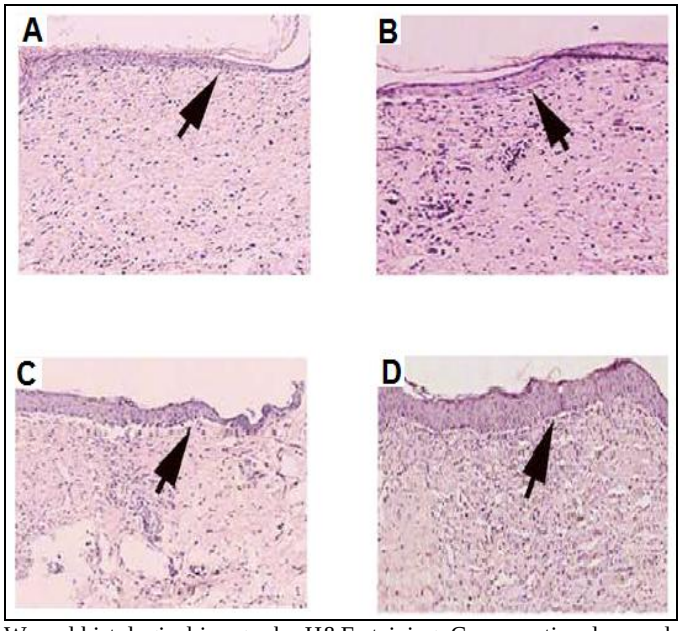


Percentage of wound of each group in STZ induced diabetic mice (n = 6).

Fig 4: Percentage of wound healing by silver nanoparticles from *A. indica*

3.3.3. Histopathology

To evaluate the effect of extracted silver nanoparticles has on early wound repair, qualitative patterns of collagen deposition were assessed after thermal wound. In sections stained with Gomori's trichrome, wounds that were not treated show pale green staining in the adjacent tissue that is indicative of a wide degree of disruption to the collagen deposition patterns in the dermis immediately adjacent to the wound site. Skin without wound shows the staining indicative of normal non-disrupted collagen-deposited patterns. Additional differences were evident between the control wounds and treated wounds within the surface epithelium. Treated with nanoparticle, the wound show significantly less epidermal hyperplasia (31.9±1.7mm) than control wound (75.2±6.2mm). Granulation tissue in the treated thermal wound beds also had a higher cell density (approximately 19 cells per 1,000mm^2) compared with control wounds (approximately 10 cells per 1,000 mm^2) (Plate 5).



Wound histological images by H&E staining. Cross-sectional wound areas were indicated by arrows. A-Control, B-SNP, C-SSD, D-Untreated wound.

Plate 5: Histochemical Analysis

Silver has been used in the clinical setting as an antimicrobial for over a century, and silver nitrate is still a common antimicrobial used in the treatment of chronic wounds. Silver nitrate causes a significant amount of staining of virtually any surface with which it comes into contact and can also cause irritation to tissues. Silver sulfadiazine was introduced in the 1960s to overcome some of the shortcomings of silver nitrate, but both are limited in the clinic due to the need for a high frequency of application, inactivation of much of the silver by wound fluid and the formation. New silver-impregnated dressings were designed to overcome these limitations, in particular the rapid inactivation of silver. In these dressings, as silver is consumed by interaction with target cells or inactivated by protein.

The biological effects of AgNPs on wound healing appear to be significant. The study with performed experiments using an excisional wound model, would able to show that AgNPs could exert differential effects on keratinocytes and fibroblasts during healing. AgNPs on the one hand, could promote wound healing through facilitating the proliferation and migration of keratinocyte, on the other hand, they could reduce the formation of collagen by fibroblasts by driving their differentiation into myofibroblasts [14].

In addition to this significant finding, AgNPs were also shown to facilitate wound healing through modulation of various cytokines. Using a contaminated wound model in pigs, Xu and groups [15] found accelerated healing was characterized by rapid development of well vascularized granulation tissue that supported the tissue that supported the tissue grafting after injury.

4. Conclusion

The data conclude the study signifies the metal nanoparticle that will allow us to deep in the design of new drug delivery systems with potential to improve the clinical efficacy of the therapeutic effect.

4.1 Conflict of interest

The authors do not have any conflict of interest.

5. Acknowledgements

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6. Reference

1. Chandrasekharan N, Kamat PV. Improving the photo-electrochemical performance of nanostructured TiO_2 films by adsorption of gold nanoparticles, J Phys Chem. 2000; 104:10851-10857.
2. Kumar SA, Abyaneh MK, Gosavi SW, Kulkarni SK, Pasricha R, Ahmad A *et al*. Nitrate reductase-mediated synthesis of silver nanoparticles from $AgNO_3$ Biotechnology Letters. 2007; 29:439-445.
3. Marie-Christine Daniel, Didier Astruc, Gold Nanoparticles, Assembly, Supramolecular Chemistry, Quantum-Size-Related Properties, and Applications toward Biology, Catalysis, and Nanotechnology, Chem Rev. 2004; 104:293-346.
4. Otari SV, Patil RM, Ghosh SJ, Thorat ND, Pawar SH. Intracellular synthesis of silver nanoparticle by actinobacteria and its antimicrobial activity (Spectrochimica Acta Part A: Molecular and Biomolecular Spect. 2015; 136:1175-1180.
5. Jean M, Feugang Ramey, Youngblood C, Jonathan M, Greene Abed, Fahad S *et al*. Application of quantum dot nanoparticles for potential non-invasive bio-imaging of mammalian spermatozoa, J Nanobiotech. 2012; 10:45.
6. Saxena A, Tripathi RM, Singh RP. Biological synthesis of silver nanoparticles by using onion (*Allium cepa*) extract and their antibacterial activity, Digest J Nanomaterials and Biostructures. 2010; 5(2):427-432.
7. Sun X, Luo Y. Preparation and size control of silver nanoparticles by a thermal method, Mater Lett. 2005; 59:3847-3850.
8. Sidhu O, Vishal Kumar P, Hari MB. Variability in triterpenoids Nimbin and Salanin composition of neem among different provenances of India Ind crops products. 2004; 19:69-75.
9. Chandran SP, Minakshi Chaudhary, Renu Pasricha, Absar Ahmad, Murali Sastry. Synthesis of gold nanotriangles and silver nanoparticles using Aloe vera plant extract. Biotech Prog. 2006; 22:577-583.
10. Geethalakshmi E, Sarada DV. Synthesis of plant-mediated silver nanoparticles using Trianthema decandra extract and evaluation of their antimicrobial activities. International, J Eng Sci and Tech. 2010; 2:970-975.
11. Roy N, Barik A. Green synthesis of silver nanoparticles using unexploited weed resources, Int J Nanotech Appl. 2010; 4:95-101.
12. Magudapatty P, Gangopadhyayrans P, Panigrahi BK, Nair KGM, Dhara S. Electrical transport studies of Ag nanoparticles embedded in glass matrix, Physica B. 2001; 299:142-146.
13. Song H, Jang BS Kim. Biological synthesis of gold nanoparticles using Magnolia kobus and Diospyros kaki leaf extracts, Process Biochem. 2009; 44:1133-1138.
14. Fong J, Wood F, Fowler B. A silver coated dressing reduces the incidence of early burn wound cellulitis and associated costs of inpatient treatments: Comparative patient care audits Burns. 2005; 31:562-567.
15. Xu C, Xu F, Wang B, Lu T. Electrospinning of poly (ethylene-co-vinyl alcohol, nanofibers encapsulated with ag nanoparticles for skin wound healing, J Nanomat. 2011; 61(9):2116-2119.