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EVALUATION OF WOUND HEALING ACTIVITY OF BIOSYNTHESIZED SILVER NANOPARTICLES FROM AQUEOUS EXTRACT OF *CASSIA AURICULATA L.*

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ABSTRACT

In this present study, biosynthesis of AgNp's from aqueous extracts of *C. auriculata* and its wound healing activity was documented. The synthesis of AgNp's was achieved by treating $AgNo_3$ solution with aqueous extract of *C. auriculata*. The formations of AgNp's were confirmed by a color change of the solution from watery to brown color. The active phytochemicals present in the plant was responsible for the immediate reduction of silver ion (Ag+) to metallic silver nanoparticles (Ag^o). The reduced AgNp's were characterized by Transmission Electron Microscope (TEM), Scanning Electron Microscope (SEM), FTIR, UV–vis spectroscopy. The spherical shaped AgNp's were observed and it was found to be 15-90 nm range of sizes. The particle size range of AgNp's monitored by particle size analyzer Mastsizer 2000 and result was found to be 0. 257µm. The wound healing activity of biosynthesized AgNp's was studied by excision and incision wound models. In the excision wound model the AgNp's showed increased the percentage of wound contraction than standard drug Povidone iodine ointment and aqueous extracts of *C. auriculata* treated group of animals. For incision wound model AgNp's showed maximum rate of tensile strength when compared to standard drug and aqueous extract of *C.auriculata* treated group of animals. The results suggest that biosynthesized AgNp's from aqueous extracts of *C. auriculata* posses a significant wound healing potential in normal wound.

Key words: Green synthesis, silver nanoparticles, Characterization, UV-Vis, TEM, SEM.

INTRODUCTION

Today, nanotechnology is a commonly used buzzword in several fields of science and recently in drug delivery. The researchers in the field of nanotechnology are coming up, that metal nanoparticles have all kinds of unexpected benefits in both the conventional technology and medical industries (Dosch H *et al.,* 2001). Among noble metal nanoparticles, Silver nanoparticles (AgNp's) have broad application in biomedical science (Ram Prasad *et al.,* 2013). The AgNp's have been synthesized many ways described in various literatures, which include physical, chemical, and biological methods. The physical and chemical methods are several in numbers, and many of these methods are leading to adverse effect due to the presence of some toxic chemicals, expensive and potentially dangerous to the environment(Shalini Chauhan *et al.,* 2011). The Biological methods of synthesis of nanoparticles such as microorganism, enzymes, fungus and plant extracts have been suggested as possible Eco- friendly alternative methods for chemical and physical methods (Panneerselvam C *et al.,* 2012).

Wounds are visible results of individual cell death or damage. It is the disruption of the cellular, anatomical and functional continuity of living tissue, produced by physical, chemical, electrical or microbial insults in the tissues (Jagtap *et al.,* 2009). Wound healing is the process of repair that follows injury to the skin and other soft tissues (Swati Rawat *et al.,* 2011) .Wound care and maintenance involve a number of measures such as dressing and administration of painkillers, anti-inflammatory agents and healing promoting drugs, etc. It runs through a number of phases such as coagulation,

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inflammation, granulation, fibroplasias, collagenation, wound contraction and epithelialization. Many different synthetic drugs are used for the treatment of wound healing in modern medicine. Moreover, herbal medicines are crucial in wound healing since they initiate disinfection, debridement and provide a moist environment for a natural healing process (Zouhir Djerrou *et al.*, 2010).

Silver metal and its compound have been known to have strong inhibitory and bactericidal effects as well as a broad spectrum of antimicrobial activities. It has been used for centuries to prevent and treat a variety of diseases, including pleurodesis, cauterization, and healing of skin wounds. Silver metal slowly changes to silver ions under our physiological system and interact with bacterial cells, thus silver ions will not be so high enough to cause normal human cell damage (Cho KH *et al.*, 2005).

Cassia auriculata L. Commonly known as *Tanners Cassia*, also known as "Avaram" in Tamil. It is a shrub that belongs to the family *Caesalpiniaceae* (Devados Kumarasamy Raja *et al.*, 2013) . Most of the plants of this genus *Cassia* are well known in Indian system of medicine for their cathartic, purgative and antibiotic properties. It is also good for ulcers, leprosy, skin diseases (Gaikwad *et al.*, 2010). The tea prepared from the leaves is used in chronic fever, flowers and pods are used in diabetes and urinary disorders. Very little research was reported on wound healing activity of *C. auriculata* AgNp's. Hence, the present work was undertaken to assess the wound healing activity of biosynthesized AgNp's from aqueous extracts of *C. auriculata L.*

MATERIALS AND METHODS

Collection and Drying of plant materials

The AR grade silver nitrate ($AgNO_3$) was purchased from Sigma –Aldrich Chemicals. The Healthy aerial parts of the *C. auriculata* (stem, leaves, flowers and seeds) were collected from the herbal garden, Faculty of Agriculture, Annamalai University, Annamalai Nagar, Tamil Nadu. The collected plant was authenticated by Mr.V.Chelladurai (Retired) Research Officer-Botany; Central council for research in Ayurveda & Siddha, India and voucher specimens (No. 1957) were kept in the Pharmacognosy Lab, Department of Pharmacy, Annamalai University for future reference. The plant was washed thoroughly three times with purified water and once with distilled water. The plant materials were air shade dried and then powdered using electric blender to make a coarse powder. The powdered samples were kept in sealed containers for extraction purposes.

Synthesis of silver nanoparticles

The aqueous extract of *C. auriculata* was used for the reduction of Ag^{+} ions to Ag^{O}. It was prepared by taking 10 mg of dried plant materials in 500 ml Erlenmeyer flask along with distilled water and then boiling the mixture for 5min. The extract was filtered with Whatman No. 1 filter paper and stored at -15^{0}c and could be used for within one week. The filtrate was treated with an aqueous 1mM AgNO3 solution in an Erlenmeyer flask and incubated at room temperature. As a result, a brown colored solution was formed, indicating the formation of AgNp's. It showed that aqueous silver ions could be reduced by aqueous extract of plant parts to generate extremely stable AgNp's in water. The colored AgNp's solution was centrifuged at 10,000 rpm for 10 min, the supernatant liquid was decanted. The resulting suspension was re dispersed in 10 ml sterile distilled water and centrifugation process was repeated for three times. Thereafter, the purified suspension was used for characterization of AgNp's (Umesh B. Jagtap *et al.*, 2013).

Characterization of silver Nanoparticles (AgNp's)

UV-Visible spectrum analysis was done by using UV-Visible spectrometer UV-2450, (Shimadzu). The diminution of the pure silver ion was monitored by measuring the UV-Visible spectrum of the reaction medium after diluting a small aliquot of the sample into deionized water. 1 ml of the sample was a pipette out into a test tube and diluted with 4 ml of deionized water. The presence of nanoparticles was confirmed by obtaining a spectrum in the visible range of 200 -800 nm (Ravichandran Verasamy *et al.*, 2011). The particle size range of the AgNp's was determined by using particle size analyzer, Mastsizer 2000. The particle size was determined based on the Brownian motion of the nanoparticles. Transmission electron microscopy (TEM) technique was used to visualize the size and shape of AgNp's. TEM area images were taken on Philips model CM 200 instrument operated at an accelerating voltage at 200 keV. Scanning electron microscope (SEM) analysis was carried out by a thin film of the sample which was organized on a small aluminum plate by simply dropping a very small amount of the sample on the plate, extra solution was transferred using a blotting paper and then the film on the plate was set aside to dry overnight. The SEM analysis was performed on a JEOL, model JSM-6390 instrument operated at an accelerating voltage of 20 keV and counting time of 100 s. The synthesized AgNp's were mixed with KBr pellets, and then subjected to a wide range of FTIR spectral analyses (Nicolet Avatar Model FT-IR spectrophotometer).

Experimental studies on animals

Wistar albino rats of either sex, weighing 120–150 g were used for in vivo studies, the experiment was conducted in conformity with the internationally accepted principles for laboratory animal use and the experimental

protocols were approved by the Institutional Animal Ethical Committee (IAEC), central animal house, Annamalai University, Annamalai Nagar, Chidambaram, India (160/1999/CPCSEA). The animals were housed in polypropylene cages in standard environmental conditions (20–25 °C), feed with standard rodent diet *ad libitum* and tap water. The animals were acclimatized to laboratory conditions for a week prior to the initiation of the experiment. 12 hours before the start of the experiment, rats were deprived of food, but given free access to water (WHO chronicle, 1985).

Excision wound model

The animals were anesthetized with anesthetic ether by open mask method and placed on an operation table in its natural position. An excision wound was inflicted on the dorsal thoracic region 1–1.5 cm away from the vertebral column on either side and 5 cm away from the ear. The skin impressed area was excised to the full thickness to obtain a wound area about 500 mm^2 and 2mm diameter and 2mm depth. Hemostasis was achieved by blotting a wound with a cotton swab soaked in normal saline. Animals were divided into four groups of 6 each. The group 1 was left untreated and considered as a control, group 2 was received standard drug Povidone-iodine ointment, group 3 received 1% aqueous extract of *C.auriculata* mixed with ointment base and group 4 was treated with 1% *C. auriculata* AgNp's mixed with ointment base. The ointment topically applied once daily starting from the day operation till complete epithelialization. Wound areas were measured on days 3,7,11 and 19 (Rupesh Thakur *et al.,* 2011). The wound area was drawn with permanent marker on transparent sheet .The circular marked area of the transparent sheet was excised and measuring the weight for calculating the percentage of wound contraction. Percentage wound contraction was calculated as:

$$\% \text{ of the wound closer} = \frac{(\text{Initial wound size} - \text{specific day wound size}) \times 100}{\text{Initial wound size}}$$

Incision wound model

Para vertebral straight incision of 6cm length was made through the entire thickness of the skin. On either side of the vertebral column with the help of a sharp scalpel. After complete hemostasis, the wounds were closed by means of interrupted sutures placed at approximately 1 cm apart. Animals were treated daily with drugs, as mentioned above under excision wound model from 0 days to 9 th post wounding day. The wound breaking strength was estimated at 10 the day by tensile tester (Instron 6021) (Rupesh Thakur *et al.,* 2011).

Tensile strength was calculated using the following formula:

$$\text{Tensile strength} = \frac{\text{Total breaking load}}{\text{Cross-sectional area}}$$

Statistical analysis of information:

Statistical comparison was performed using one way analysis of variance (ANOVA) and for multiple comparison versus control group was done with Dunnett's test. All statistical analysis was performed using Graph pad prism version 5. $P< 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The addition of aqueous extract *C.auriculata* to silver nitrate (AgNO3) solution resulted in color change of the solution from clear to dark brown color due to the formation of AgNp's. The Intensity of brown color increased in direct proportion to the incubation period. It may be due to the excitation of surface Plasmon resonance (SPR) effect and reduction of $AgNO_3$ (Akl M Awwad *et al.,* 2013). The UV- spectrum of *C. auriculata* AgNp's was recorded from the reaction medium. The results showed maximum absorption peak rang at 420.5 nm (Fig. 2).

Particle size distribution

The particle size range of AgNp's synthesized from *C.auriculata* monitored by using particle size analyzer Mastsizer 2000. The result showed that AgNp's average size range was found to be 0. 257µm.

Transmission Electron Microscope (TEM)

TEM technique was employed to visualize the size and shape of AgNp's. Pictures below to obtain by TEM showed, the Ag Np's synthesized from aqueous extracts of *C. auriculata.* It is observed that the morphology of the AgNp's was predominately spherical in shape. The overall morphology of the Ag Np's produced by reduction of Ag+ ions with 1mM $AgNO_3$ was composed of almost uniform Ag Np's. The typical TEM image of the biosynthesized Ag Np's from aqueous extracts of *C. auriculata* shown in Fig 4.

Scanning electron microscope (SEM) analysis of AgNp's

The Figure 5 Showed in SEM image of AgNp's prepared from aqueous extracts of *C.auriculata*. SEM provided further insight into the morphology and size details of the AgNp's. Comparison of experimental results showed that relatively spherical and uniform AgNp's were formed with diameters of 15-90 nm.

FTIR Spectral Analysis of AgNp's

FTIR measurements were carried out for indicating various functional groups present at different positions in the AgNp's synthesized by *C. auriculata* aqueous extract and spectrum was shown in Figure: 6. The synthesized AgNp's displays a number of absorption peaks, reflecting its complex nature. The band at 3445.53cm-1 corresponds to O-H stretching H-bonded alcohols and phenols. The strong absorption peak at 2924.61 cm−1 could be assigned to – CH stretching vibrations of – CH_3 and – CH_2 functional groups. Assignment at 1636.29 corresponds to C=O stretching in Amide. The absorption peaks at 1507.30 cm-1could be attributed to the presence of N-O stretching in Nitro compounds. The absorption peaks at 1457.61 cm−1 could be attributed to the presence of C= C stretching in Aromatic. The intense band at 1019 cm-1 can be assigned to the C-F stretching vibrations of Alkyl halides. From the analysis of FTIR studies, we confirm that the carbonyl group of the amino acid residues and proteins have the strong ability to bind with metals indicating that the proteins could possibly form the metal nanoparticles (i.e. Capping of AgNp's) to prevent agglomeration and thereby stabilize the medium. This suggests that the biological molecules could possibly perform the dual functions of formation and stabilization of AgNp's in the aqueous medium(Udayasoorian *et al.,* 2011).

Excision wound model

Wound contraction ability of excision model was evaluated at different time intervals till complete wound healing process. The percentage of wound contraction was measured by the weight variation of the trace paper, which was incised from the wound area marked trace paper. The Figure 7 showed the photograph was taken before starting the treatment in different groups. The Figure 8 showed the percentage of the wound contraction of different treated groups in 19th days.

Table: 1 Represent the effect of aqueous extract of *C. auriculata* and AgNp's variants on percentage of wound contraction in the excision wound model. The control rats showed a time dependent decreases the wound size from 0.01725 to 0.0050 mg from the day 3rd to 19th. The group treated with standard drug (Povidone – iodine ointment) showed 0.01463 to 0.0036 mg from the day 3rd to 19th day. 1%w/w aqueous extract incorporated with simple ointment treated group showed decreased the wound size from 0.01500 to 0.0031mg from the day 3rd to 19th day and 1%w/w AgNp's incorporated with simple ointment treated group showed decreased the wound size from 0.01313 to 0.0025mg from 3rd to 9th day. It was noticed from the results wound contracting ability of C. *auriculata* AgNp's were significantly greater than ($P < 0.05$) that of the control, and aqueous extract *C. auriculata* which was comparable to that of the reference standard Povidone-iodine ointment.

Incised wound model

The incision wound study was carried out to measure the tensile strength of the regenerated tissue. The aqueous extract of *C.auriculata* and *C.auriculata* AgNp's was studied to access the wound healing properties in Wistar albino rats of the incision wound model and the results are reported in Table: 2, in the incision wound studies, the tensile strength of AgNP's was increased on 10th past wounding day with all the samples compared with Control. The tensile strength was observed in 10th past wounding day with *C.auriculata* AgNp's found to be 86.850±0.821, aqueous extract of *C.auriculata* showed 84.315±0.768, standard drug Povidone-iodine ointment showed 86.113±0.89, control group (animals treated with simple ointment) showed 63.523±1.201 and control group (the skin samples were excised from the normal animals on experimental day) showed 106.18±1.022.

The studies from the results biosynthesized AgNp's showed better wound healing activity, then aqueous extract of *C. auriculata*, control group (treated) and wound treated with standard drug Povidone-iodine ointment. When compared with control group results the *C.auriculata* AgNp's showed almost nearly equal effects. From The results we conclude that AgNp's had a strong effect on skin wound and accelerate healing.

Table 1. Effect of wound healing activity of aqueous extract of *C. auriculata* and *C. auriculata* AgNp's on the excision wound model

Post Wounding days	Weight of trace paper (mg)			
	Control (Simple ointment)	**WStd (Povidone-iodine)**	**WExt (1%w/w of Aq. Extract of *C. auriculata***	**WFmn (1%w/w of *C.auriculata* AgNp's**
3rd day	0.01725±0.00127	0.01463±0.0096	0.01500±0.00160	0.01313±0.00934
7th day	0.0142±0.0008	0.0123±0.0008	0.0116±0.0009	0.0100±0.0001
11th day	0.01300±0.0005	0.0116±0.0004	0.01300±0.0007	0.0098±0.0009
19th day	0.0050±0.0008	0.0036±0.0004	0.0031±0.0003	0.0025±0.005*

Values are expressed as the mean ± S.D; Statistical significance (p) calculated by one way ANOVA followed by Dunnett's $P< 0.05$ calculated by comparing treated groups with the ONLY WOUND group.

Table 2. Effect of wound healing activity of aqueous extract of *C. auriculata* and *C. auriculata* AgNp's on the incision wound model

S.NO	Group	10^{th} past wounding day
		Tensile strength
1	Control	106.18±1.022
2	Control (Treated)	63.523±1.201
3	WStd	86.113±0.897
4	WExt	84.315±0.768
5	WFmn	86.850±0.821

Values are expressed as the mean ± S.D; n = 6, Statistical significance (p) calculated by one way ANOVA followed by Dunnett's $P< 0.05$ calculated by comparing treated groups with the ONLY WOUND group. The $P< 0.0001$, consider extremely significant.

Figure 1: The photograph showing color change of plant extract after adding $AgNo_3$ solution (a) aqueous extract of *C.auriculata* (b) $AgNo_3$ solution (c) synthesized AgNp's

Figure 2: UV-visible absorption spectrum of AgNp's synthesized from aqueous extract of *C. auriculata*

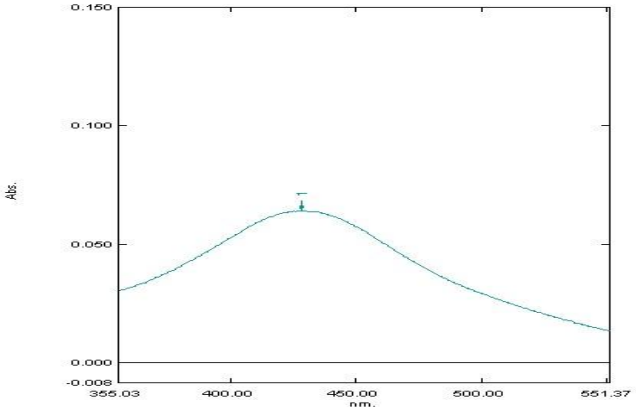


Figure 3. Particle size distribution of *C.auriculata* AgNp's

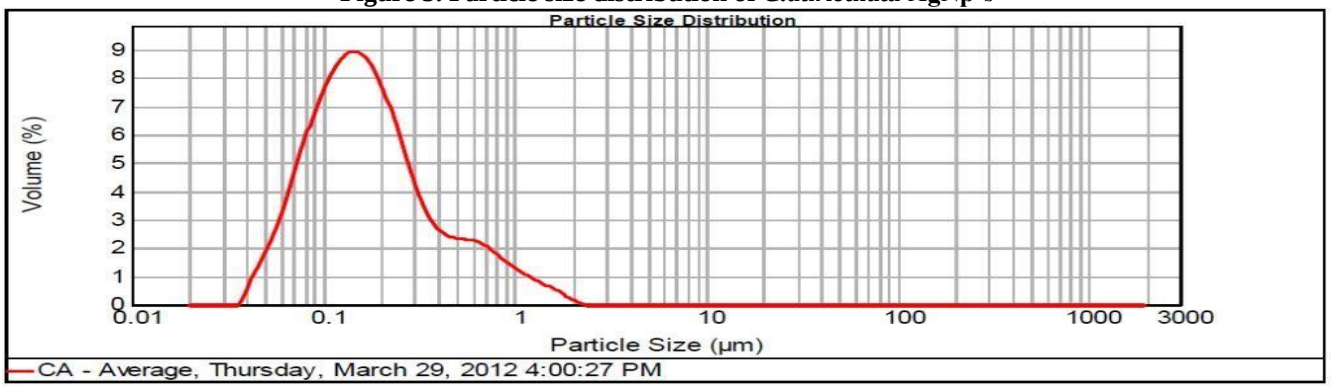


Figure 4. TEM image of AgNp's synthesized by *C.auriculata*

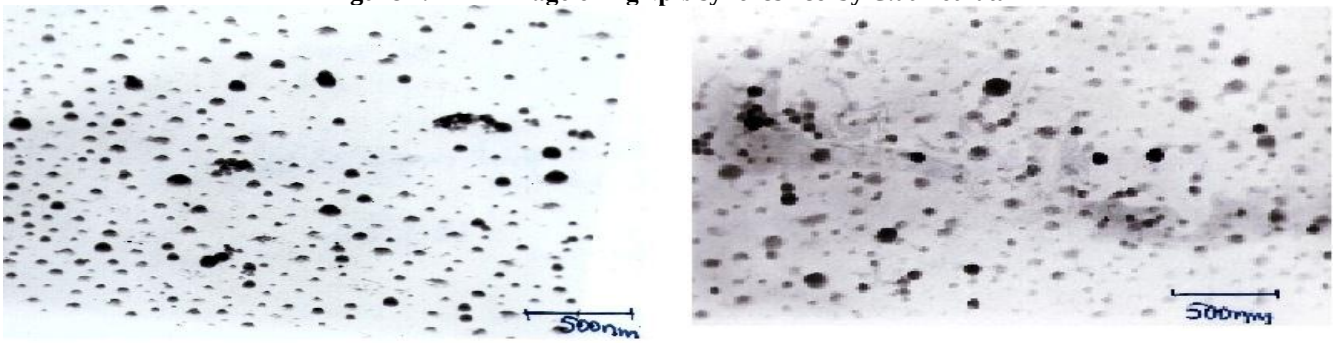


Figure 5. SEM image of AgNp's synthesized by *C.auriculata*

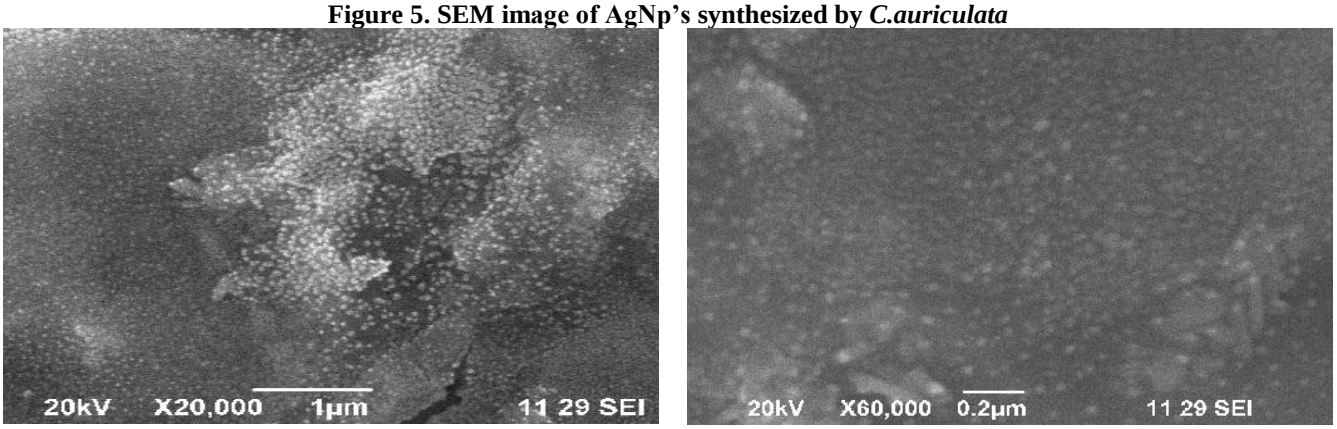


Figure 6. FTIR spectrum of AgNp's using an aqueous extract of *C. auriculata*

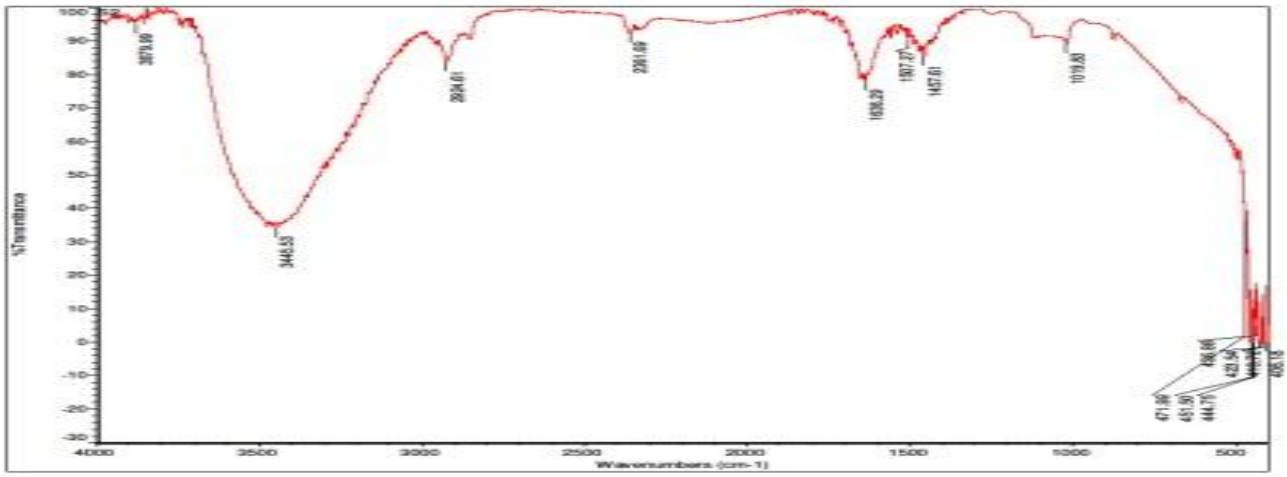


Figure 7. Excision wound formation of skin – 0 day, A- (Group I)- Normal control B – (Group II)– Povidone iodine ointment, C- Group III – Aqs. Extract of *C. auriculata*, D – Group IV – *C. auriculata* AgNp's

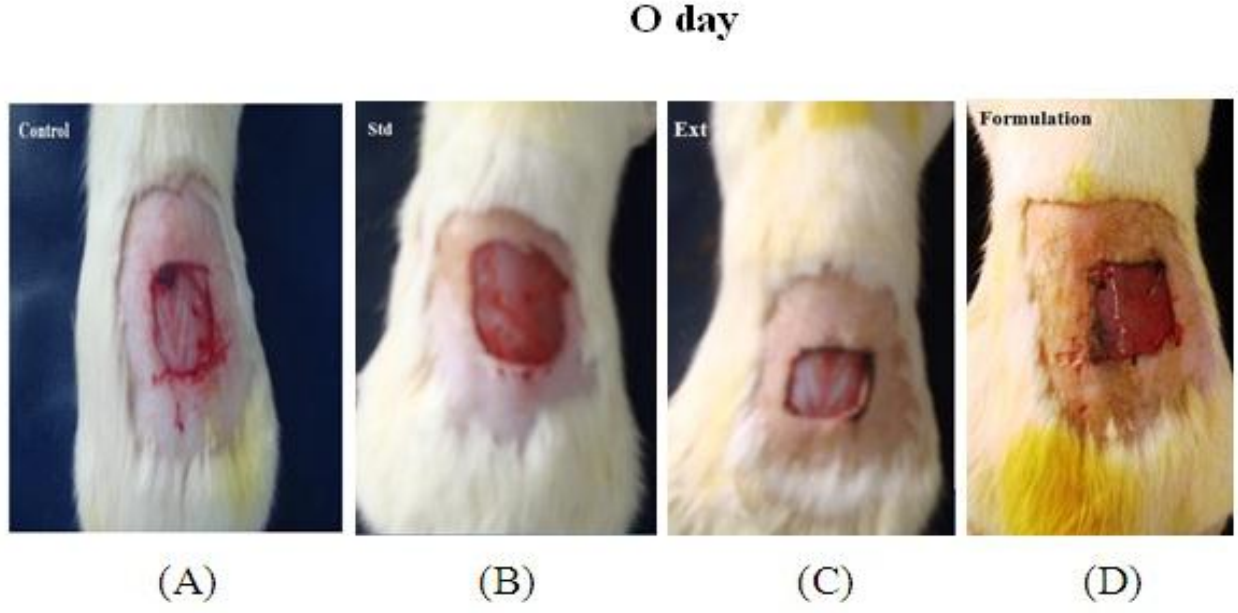


Figure 8. Macroscopic observation of excision wound on – 19th day (A- (Group I- Normal control, B – (Group II)– Povidone iodine ointment, C- (Group III)– Aqs. Extract of *C. auriculata*, D – (Group IV)– *C. auriculata* AgNp's

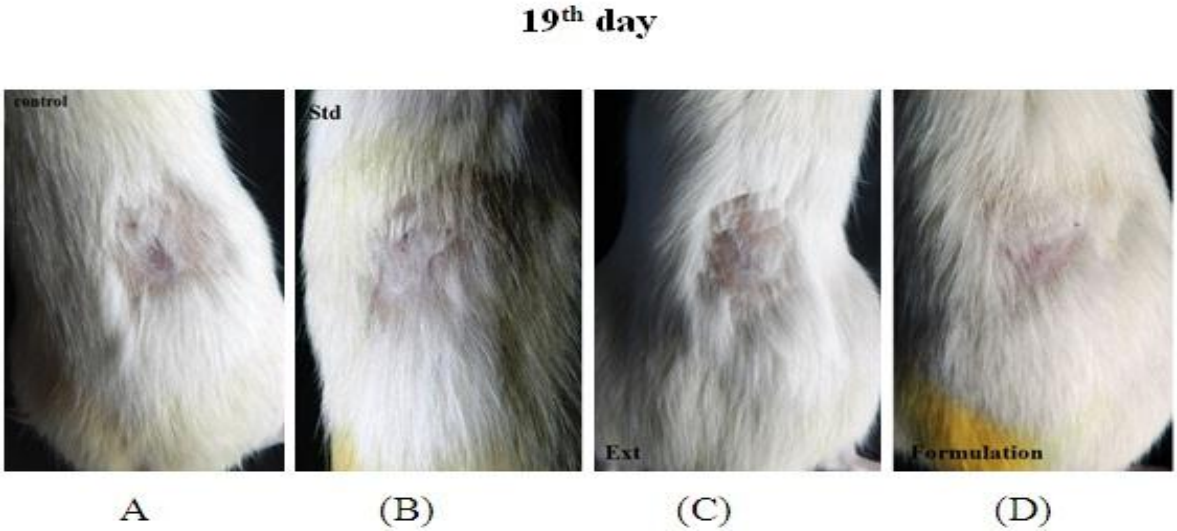


Figure 9. Percentage of wound contraction at different time intervals

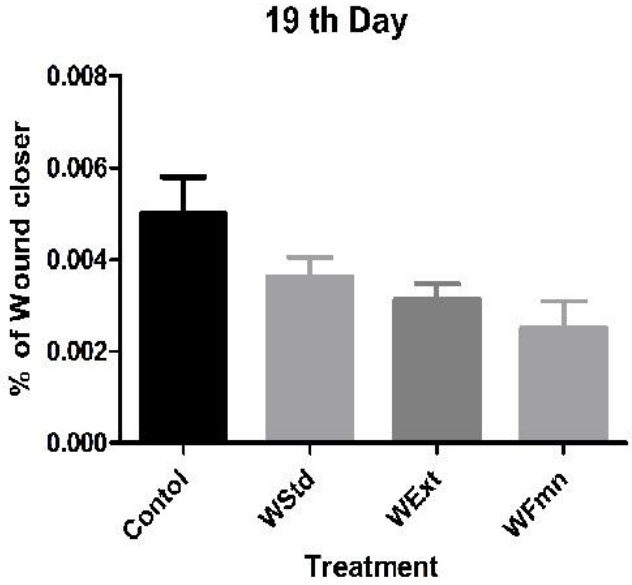
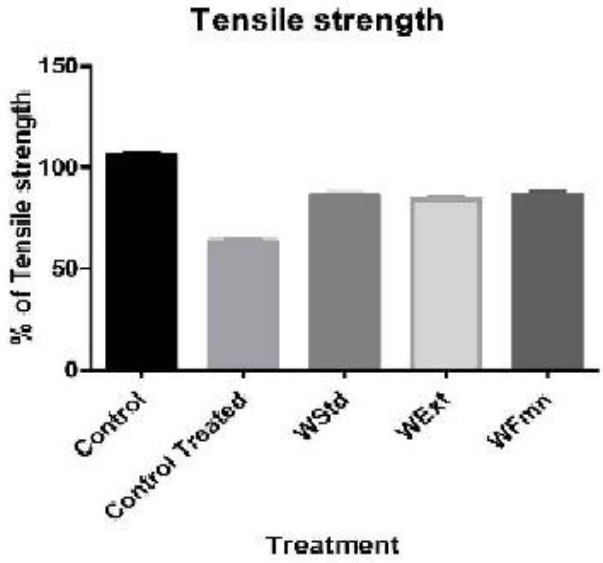


Figure 10: Percentage of Tensile strength at 10th past wounding day



DISCUSSION

Green chemistry approach towards the synthesis of nanoparticles has many advantages such as, Ease with which the method can be scaled up and economic viability. We have developed a fast, eco-friendly and convenient method for the synthesis of silver nanoparticles using an aqueous extract of *C. auriculata* with an average particle size range of 0. 257µm. It is generally the formation of AgNp's was recognized by UV-vis spectroscopy. It could be used to examine the size and shape of the nanoparticles in aqueous suspensions. AgNp's have free electrons, which give rise to an SPR absorption band, due to the combined vibration of electrons of metal nanoparticles in resonance with the light wave. Due to the excitation of plasma resonances on inter-band transitions, some metallic nanoparticles dispersions exhibit unique peaks. The broadness of the peak is a good indicator of the size of the nanoparticle. As the particle size increases, the peak becomes narrower with a decreased bandwidth and increased band intensity (Rajesh *et al.*, 2012). The morphology and size of the *C.auriculata* AgNp's were investigated by TEM and SEM analysis. The results showed that the minimum size range of *C.auriculata* AgNp's was found to be 15-90 nm. The results of FTIR spectrum study showed in figure 6, described that biosynthesis of AgNp's from aqueous

extracts of *C. auriculata* had various functional groups such as alcohols, phenols, Amide, Nitro compounds, Aromatic and Alkyl halides. A report from the earlier studies (C. Udayasoorian et al., 2011) amino acid and proteins could possibly act as a capping agent of AgNp's. Typically, the wound healing process involved many steps that include homeostasis, inflammation, proliferation, and remodeling. The progression from an injured site of a healed wound is potentially slowed or arrested by a number of different events and conditions. One of the events that impede wound healing is colonization of the wound bed by microorganisms. It may lead to a prolonged inflammatory response, once a wound becomes infected, healing is delayed. The use of antimicrobial therapy in wound care is to control the microbial colonization and subsequent proliferation thus promoting the healing of the wounds (Bhargav Bhide *et al.,* 2011).It is well known that silver metal and its compound have been known to have strong inhibitory and bactericidal effects as well as a broad spectrum of antimicrobial activities(Hasna Abdul Salam *et al.,* 2012).

In this study, wound healing activity of AgNp's carried out with two wound models, such as excision and incision model in Wistar albino rats. The excision wound healing was assessed by the percentage of wound contraction. It was observed in the macroscopic presentation in Figure 7&8. The results from the table 1 showed that *C. auriculata* AgNp's had better wound healing activity, then aqueous extract of *C.auriculata* and standard drug Povidone iodine ointment. The incisional wound model was measured by the percentage of tensile strength. The finding from the table 2 showed that the biosynthesized AgNp's exhibit better wound healing activity than treating control groups, aqueous extract of *C. auriculata* and Standard drug Povidone iodine ointment. When compared with normal group (the skin samples were excised from the normal animals on experimental day) showed nearly equal results. From the above observed results it can be suggested that bio synthesized AgNp's from aqueous extracts of *C. auriculata* may have a sufficient wound healing activity which could be useful in the treatment of excision and incision wounds.

CONCLUSION

In summary, the aqueous extract of *C.auriculata* can be converted into AgNp's by green synthesis and the resulting *C.auriculata* AgNp's showed potential wound healing activity when compared with other formulations. The biologically synthesized *C.auriculata* AgNp's can be used in the medical field for their efficient wound healing activity. As future direction cytotoxicity assays and clinical trials should be carried out to evaluate the wound healing potential, bio compatibility and clinical usability of AgNp's. Finally a marketable form of AgNp's may be released for wound healing ability.

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