EVALUATION OF BIOLOGICAL ACTIVITY OF GREENLY SYNTHESIZED SILVER NANOPARTICLES USING ALOE VERA GEL EXTRACT AS ANTIBACTERIAL AGENT *IN-VITRO* AND *IN-VIVO*

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ABSTRACT

The present study was carried out to synthesis silver nanoparticles by using *Aloe Vera* gel and evaluates antibacterial activity *in vitro and in vivo*. The synthesis and characterization of silver nanoparticles was confirmed by Ultra Violet Visible- spectrophotometer, X-ray diffraction, Fourier Transmission Infrared Spectroscopy (FTIR) and Scanning Electron Microscopy. Reduction of the Ag+ to Ag0 during exposure to the *Aloe Vera* gel extract was followed by color change of the solution from colorless, yellow to dark brown within 24 hours. It is observed that surface Plasmon resonance peaks of the maximum absorbance of silver-nanoparticles occur at 425 nm, indicating that AgNPs were produced. Later on, using agar well diffusion and tube dilution method against pathogenic methicillin resistance *staphylococcus aureus*, (MRSA). *Pseudomonas. aeruginosa* and *Escherichia coli*, the antimicrobial properties of synthesised AgNPs were investigated. To confirm *in-vivo* antibacterial activity, after inducing complicated skin and soft tissue infection in mice by injecting *S.aureus* subcutaneously. 16µg/gm AgNPs were applied skin infected daily for three days. Silver nanoparticles were as succeeded to reduce the lesion volume in infected mice and reduce the acute inflammation symptoms as clindamycin, In conclusion, A new approach can be used to combat serious infections caused by MRSA by *Aloe Vera* AgNPs.

Keywords: inflammation, MRSA, AgNPs, soft tissue. clindamycin

تقييم الفعالية الحيوية لدقائق الفضة النانوية المغلفة حيويا من خلاصة جل الصبار كمضاد للجراثيم في الزجاج والحي

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المستخلص

أجريت هذه الدراسة لتخليق الجسيمات النانوية الفضية باستخدام جل الصبار وتقييم نشاطه المضاد للجراثيم في المختبر وفي الجسم الحي. تم تأكيد تخليق وتوصيف الجسيمات النانوية الفضية بواسطة مقياس الطيف الضوئي فوق البنفسجي المرئي والأشعة السينية، مطياف الأشعة تحت الحمراء والمجهر الإلكتروني. اختزال ايونات الفضة أثناء التعرض لمستخلص جل الصبار عن طريق تغيير لون المحلول من اللون الأصفر إلى البني الداكن في غضون 24 ساعة. لوحظ أن أعلى امتصاص للجسيمات النانوية الفضية تحدث عند 425 نانومتر، مما يشير إلى أنه تم إنتاج دقائق الفضة النانوية. بعد ذلك تم التحقق من خصائصه المضادة للبكتريا باستخدام طريقة النشر في الأطباق والتخفيف بالأنابيب ضد المكورات العنقودية الذهبية المقاومة للمثسلين، الزائفة الزنجارية والأشريكيا القولونية. ولتأكيد النشاط المضاد للبكتيريا في الجسم الحي تم بعد إحداث التهاب الجلد والأنسجة الرخوة في الفئران عن طريق حقن المكورات العنقودية الذهبية تحت الجلد. تم تطبيق دقائق الفضة النانوية 16ملغم/غم على الجلد المصاب يوميًا لمدة ثلاثة أيام. نجحت الجسيمات النانوية الفضية في تقليل حجم الآفة في الفئران المصابة وتقليل أعراض الالتهاب الحاد كما في الكليندامايسين، بذلك يعد تصنيع دقائق الفضة النانوية باستخدام جل الصبار نهج جديد لمكافحة الالتهابات الخطيرة التي تسببها المكورات العنقودية الذهبية المقاومة للمثيسيلين.

كلمات مفتاحية: الالتهاب MRSA, AgNPs . الأنسجة الرخوة. كليندومايسين

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INTRODUCTION

Intact skin provides protection from the external environment by serving as a physical barrier and maintaining a normal flora that is not conducive to the growth of pathogenic organisms. Skin and soft tissue infections (SSTIs) are common in outpatient clinic and emergency department visits and include a wide variety of infections of the epidermis, dermis, subcutaneous tissue, fascia and muscle (11,12). They are generally classified into two categories: purulent infections (e.g., furuncles, carbuncles, abscesses) no purulent infections (e.g., erysipelas, cellulitis, necrotizing fasciitis). They are then further classified into three subcategories: mild, moderate, and severe (42). The SSTIs usually result from traumatic, surgical or healthcare related skin break down with secondary inflammatory microbial invasions (10).The severity of SSTIs ranges from mild superficial to deeper or potentially fatal necrotizing infections requiring hospitalization or intensive care (17). In recent studies more than 50% of all SSTI were caused by *S. aurous* (46). Methicillin resistant *Staphylococcus aureus* (MRSA) has complicated management of cSSTIs. These pathogens, previously confined to healthcare settings, are increasing commonly in the community (8,16,33). Nanoparticles and nano devices have the potential to show a positive impact on human health and they can be used in the treatment of various serious diseases because of their activity, lower pore size and confined size. They are so technically designed that they can interact with sub-cellular levels of the body with high specificity (4, 29). The nan biotechnology is at the interface of different multidisciplinary approaches where in chemistry, biology and biophysics, congregate (13). A green synthesis is an environment-friendly approach for manufacturing well-categorized nanoparticles. One of the most deliberated methods is the production of metal nanoparticles with the help of biological organisms. Plants appear to be the most influential and appropriate platform for large-scale biosynthesis of nanoparticles (1, 21, 30, and 45). This method is more suitable and with the help of this method, the level of synthesis is quicker as compared with the case of microbial synthesis. Plants or plant-derivative resources for the biosynthesis of metal nanoparticles have guided researchers to examine contrivances of metal ions approval and bio reduction by plants, and to recognize the potential mechanism of metal nanoparticle development (20). These medicinal plants are considered as a rich source of ingredients which can be utilized as a part of drug development and synthesis (30,40). *Aloe Vera* belongs to the family of Liliaceae, it's used in traditional medicine (27, 37). It is also used in the treatment of different diseases, particularly thoses associated with skin issues and wounds (7). The leaves of *Aloe vera* have two products. the bitter yellowish juice on the outside of the inner layer just before the external covering and the gel on the inner side of the leaf *Aloe vera* gel is made up of 98.5 percent water and it‘s quite viscous in the gel form because of the sugar of glucomannan. This gel contains about two hundred active ingredients like minerals, lipids, vitamins, proteins, polysaccharides and amino acids (9, 38). some compounds can be noted like brady kinase which has anti-inflammatory characteristics and acemannan the one that can strengthens the immune system and magnesium lactate that lessens itching as well as soothing and anti-inflammatory such asantiprostaglandins and sialic acid (39). *Aloe Vera* shows a beneficial effect by reducing the inflammation significantly and providing a more mature granulation tissue which could accelerate healing and might produce a sound well-remodeled scar (19). Several other medicinal effects of *Aloe vera* has been reported including anti-inflammatory, antitumor, laxative, antiseptic, antidiabetic, antioxidant, antibacterial and antiviral activity (35). This study was aimed to synthesized silver nanoparticles by using *Aloe vera* gel and evaluates antibacterial activity *against* methicillin resistant *Staphylococcus aureus in vitro and in vivo*.

MATERIALS AND METHODS

Synthesis of silver nanoparticles

Aqueous extracts of *A. vera* was prepared by the *Aloe vera* gel (figure 1). Plant stem was cleaned by washing with distilled water and 100 gram of thoroughly washed *Aloe vera* gel was finely cut and boiled in 100 mL of sterile

distilled water. The resulting extract was filtered and stored at 4°C for further experiments (21). $AgNO_3$ (0.5 M) was prepared by taking17g AgNO3 dissolved in 200 ml of deionized water underneath magnetic stirring at room temperature for thirty minutes (25). Afterwards, 45 ml of 0.5M $AgNO_3$ was mixed with 5 ml aqueous plant extract and left until the color was changed to dark brown.

Figure 1. *Aloe vera* plant and gel

Characterization and identification of Silver Nanoparticles (AgNPs)

The formation and stability of AgNPs was seen with UV visible spectrophotometer apparatus in wavelengths between(350-700) nm. For the spectral analysis of UV-Vis. the solution absorbance was measured at one hour intervals that ranged from 1-24 hours. (1,2). the developed AgNPs solution was centrifuged for 30 min at 10,000 rpm. The AgNPs solid residues were twice washed with de-ionized distilled water and then dried at 80ºC in order to get powder AgNPs that is used for XRD (X-ray powder diffraction). then on (Shimadzu XRD-6000) with copper radiation (Cu Kα, 1.5406 Å) at 40 kV and 30 mA patterns were recorded (6,27). FT-IR measurements were done using (FT-IR Spectroscopy, ABB-Specro-Lab-MB3000, UK) that ranged from 3500 cm-1 to 500 cm-1. Then air drying was done on the silver nanoparticles pellets. These dried nanoparticles were then mixed with potassium bromide (KBr) in order ro make thin pellets and then were used in FT-IR analysis on transmittance mode (22). the aqueous solution samples of AgNPs was scanned with scanning electron microscopy (SEM) was prepared by setting a one drop of the on the carbon-covered copper grids and the films on the SEM system permitted standing for two minutes, after that there is removing of the extra solution by using a blotting paper and drying the grid. The size appropriation of the subsequent nanoparticles evaluated on the basis of SEM micrographs (41).

Test organisms: Methicillin resistance *staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli* isolate were obtained from the College of Veterinary Medicine/ Department of Zoonotic diseases/Baghdad University. These isolates spp. were identified by studying morphological and some biochemical characteristics.

Microbiological evaluation

Micro-dilution assay: The MICs of AgNPs were determined by using the tetrazolium salt in the tube dilution assay and this method was modified from (29,44). Standardized bacterial suspension (1.5×10^8 cell/ml) adjusted with 0.5McFarland tube. MIC values of AgNPs and clindamycin were determined using a 96-well sterile microtiter plate based on the micro-well dilution method (47). Biosynthesised AgNPs were serially diluted in Mueller Hinton broth at different concentrations (0.5–1024 µg/ml), (0.125-64) µg/ml for clindamycin and 10 µl of bacterial suspension was added in a final volume of 100 µl in each well. Three wells of bacterial suspension (without any drug) were considered growth control. After 24 h incubation 37˚C without shaking to visually differentiate between the live and dead cells, 200 µL of TTA reagent was added to each tube and observed for color development after 2 hr. The lowest concentration of AgNPs at which no color was seen was regarded as the MIC. Minimal bactericidal concentration (MBC) was detected by plotting 5 µl of clear-well samples into nutrient agar plates. MBC was defined as the point at which there was no microbial growth.

Well-diffusion assay: Antibacterial activity was checked by agar well diffusion method. Fresh overnight grown culture was used for inoculums preparation. Inoculums were prepared in peptone water and incubated for 2 hours. Turbidity was adjusted equivalent to 0.5 McFarland units (10^8 CFU/ml). Volume of the suspension was altogether blended to sterile Mueller Hinton agar. The agar was dispersed

into Petri dishes, after solidification, making 4 wells for plates. (6 mm) in diameter, after that wells were loaded with 0.1 ml of AgNPs concentration (4-64) µg/ml and 20 µg/ml for clindamycin. The plates were then put in the incubator in upright position at 37 C^o for 24 hours (36). Three plates were made for each concentration and the strength of these antibiotics was dictated by measuring the inhibition zone diameter around every well against the tested bacteria.

Experimental animals and inducing infection: Forty healthy male Swiss mice (White BALB/c), aged 8-12 weeks, and weights 25-30 gm.After the adaptation period, each mouse was anesthetized by isoflurane inhalation. Preparing the left flank region by clipping the hair with electrical clipper and shaving after that removing the hair by sterile gauze. Bacterial inoculums used to induce infection (skin infection) was (2.7 x 10^6cfu/ml) of MRSA suspension, 0.25 ml was s/c administered into the left flank of each mice and watched for symptom of inflammation (5).

Experimental design Forty mice were divided equally into four groups (10 mice /each, and treatment began after 24 hrs. after inducing complicated skin and soft tissue infections). Group (A): negative control, Group (B): positive control, Group (C): infected with MRSA and treated with AgNPs 16 mg/gm applied on infected skin i daily for 3 days and Group (D): infected with MRSA and treated with clindamycin 2% applied for 7 days.

Estimation of skin lesion volume

To evaluate the antibacterial effect of AgNPs and clindamycin on skin and soft tissue infection, skin lesion volume was measured by digital caliper 48 h after infection, at 2nd day of treatment and at the end of treatment in day 7th.Evaluation of treatment on lesion size was not blinded. A lesion volume score was calculated from the following equation;

$LV=(\pi/6)(L\times W2$): where LV = lesion volume. L=length of the lesion in mm. `W=width of the lesion in mm. (45).

RESULTS AND DISCUSSION

Separation and Identification of Silver Nanoparticles

UV–visible absorbance analysis: AgNPs formation was monitored with UV-Vis spectrum and color change. After 30 minutes of reaction, the reduction of silver ions into AgNPs started. and after almost 24 hours it was completed. A sharp singlar surface plasmon with resonance band of 425 nm consists the absorption spectra of AgNPs solution (Figure 2).

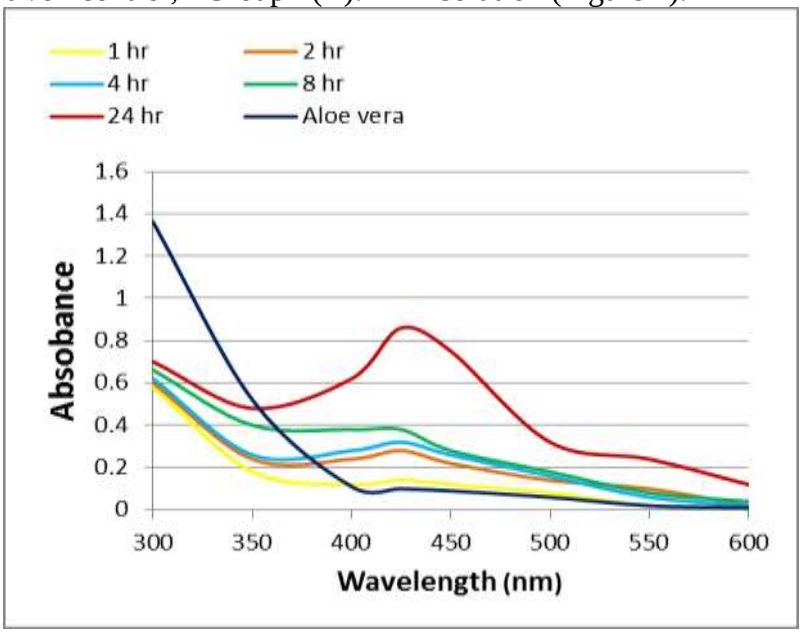

Figure 2. UV-Vis absorption spectra of AgNPs synthesized by *aloe Vera* gel at different times and color change during synthesis of silver nanoparticles

X-Ray Diffraction (XRD): XRD is an analytical technique that is very rapid and is used in synthesized nanoparticles characterization of crystalline material. It can also provide data on cell dimensions of units . The XRD pattern revealed seven sharp peaks

(27.56°, 29.80°, 32.90°, 46.82°, 55.05°, 57.81°, 77.60°) indicating the structure of silver nanoparticles as it ranged from 20 to 100 in the whole spectrum of 2θ (Figure 3A). Using Scherr's equation, the silver nanoparticles average particle size of was estimated as (25.26) nm.

Fourier transform infrared spectroscopy (FT-IR): To identify the functional groups, FTIR analysis was done, between $AgNO_3$ and the reducing agent (*aloe vera gel*). their role was predicted in the silver Nanoparticles synthesis. N–H stretch was seen in the absorption bands 3328 however it may also belong to alcohols and phenols with stretching vibration of intermolecular hydrogen bonded O–H groups, aldehydic C–H stretching was seen at 2936, and carbon dioxide was seen at the2360 cm−1 bands. While the bands 1640, 1384, 1088, 1024 mean C–O or N–H, C=O, C–N stretching, C-O group stretching, respectively (Fig. 3B).

Scanning electron microscopic analysis (SEM): The prepared AgNPs size and shape seen with SEM imaging are shown in the (Figure 3C). Different magnifications of SEM images of Ag-NPs show agglomeration of the particles, and the AgNPs particles look almost like a spherical shape with a 25.26nm of mean average diameter of 25.26nm.

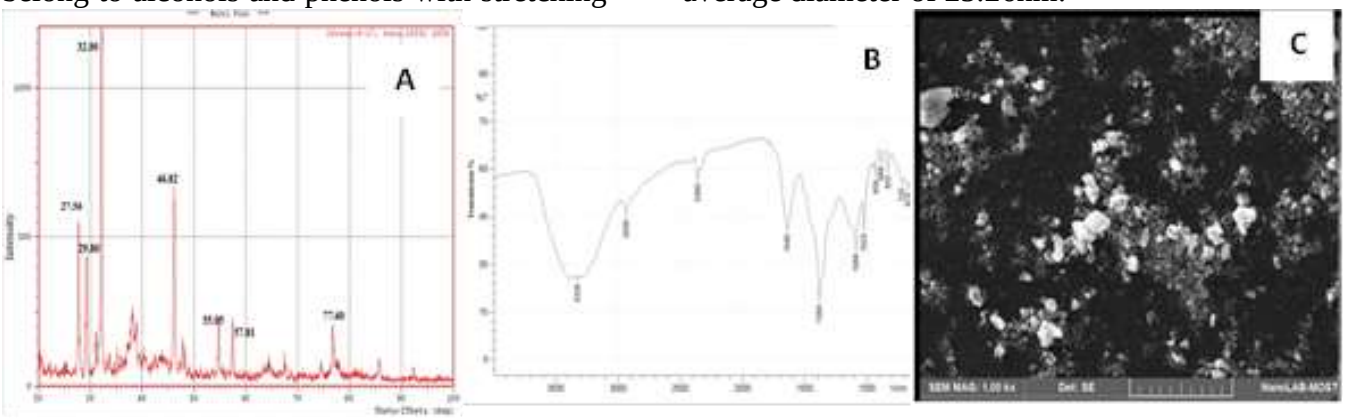

Figure 3. A: XRD patterns, B: FTIR spectra, C: SEM image of silver nanoparticles synthesized by *aloe Vera* gel

Antibacterial activity of *aloe vera* silver nanoparticles

Silver nanoparticles of *Aloe vera* showed inhibition zones ranging from (7-20) mm against different reference strains. The effect of different concentrations of silver nanoparticles against bacteria is given in Table 1. AgNP showed greater inhibition zone (20 mm) against *E. coli* more than positive control. MIC values for silver NPs against different bacterial strains ranged from 96 to 120 µg/ml. Silver NPs showed minimum MIC values against *E. coli, and P. aeruginosa* while maximum against *S. aureus* (Figure 4).

Table 1. Antibacterial effect of *Aloe vera* silver nanoparticles against bacterial strains

Bacterial Strains	Zone of inhibition in (mm) at different concentrations					
	AgNP 4µg/ml	AgNP 8µg/ml	AgNP 16µg/ml	AgNP 32µg/ml	AgNP 64µg/ml	Clindamycin 20µg/ml
E. coli	**8 ± 0.16**	**10 ± 0.52**	**12 ± 0.14**	**16 ± 0.35**	**20 ± 0.57**	**18 ± 0.21**
P. aeruginosa	**7 ± 0.18**	**9.2 ± 0.36**	**11 ± 0.20**	**13 ± 0.25**	**16 ± 0.19**	**17 ± 0.36**
S. aureus	**7 ± 0.12**	**8.5 ± 0.11**	**12 ± 0.23**	**14 ± 0.17**	**19 ± 0.10**	**18 ± 0.12**

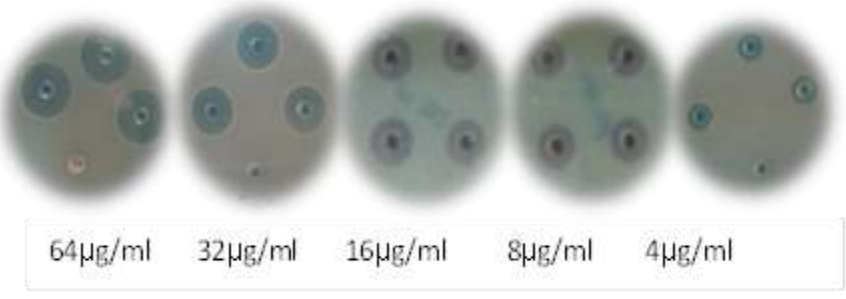

Figure 4. Inhibition zone of AgNPs at different concentration

The effect of AgNPS might be due to the small size of synthesized AgNPs as compared to the plant extract that enables them to penetrate into the thick walls of bacterial strains (28,36). Bactericidal activity of the synthesized nanoparticles was higher against gram-negative bacteria than against gram-positive bacteria. It is mainly because of the fact that the cell wall of the gram-negative bacteria contains less peptidoglycan as compared to the gram-positive bacteria (24). Further reports also support the fact that nanoparticles have more bactericidal effects for gram-negative bacteria than gram-positive ones due to the thicker peptidoglycan cell wall (28). The size and shape of nanoparticles may affect antimicrobial activity. Even the stabilizing agent used may also affect the antibacterial activity, as well as hydrophobicity and charges of the particles, also can affect it. The smaller sized silver nanoparticles present the maximum antimicrobial activity. It was also observed that the smaller sized spherical shaped silver nanoparticles confirmed an improved antibacterial activity as compared to the larger and triangular shaped silver nanoparticles because they have the greater surface area to volume ratio which makes them more reactive than other shaped particles (30,45). The shape and size dependent bactericidal efficacy could simplify a new pattern for making an allowance for the true role of silver nanoparticles as antimicrobial agents in drug design (1).when AgNPs interact with microorganisms, silver ions (Ag+) are released and these ions may affect and damage the microorganism in different ways; for example, they attack the negatively-charged cell walls of the microbes to deactivate cellular enzymes and disrupt membrane permeability; consequently, cell lysis and cell death occurs (14). Furthermore, a number of mechanisms have been attributed to the antimicrobial activity of silver NPs. The AgNPs may alter the membrane permeability of the microbes allowing inflow of AgNPs into the cell. The interaction of the NPs with intracellular proteins, particularly sulphur containing membrane proteins and microbial DNA can interfere with cell division leading to cell death. Replication of microbes is compromised due to the release of Ag ions from the AgNPs (15). Interestingly, the antimicrobial activity of nano-sized silver particles was found size- and shape-dependent, one of the reasons could be that different morphologies provide different areas to interact with microbes and thus results in different antibacterial efficiency (43).

Pathological gross examination

The gross examination performed to all groups infected with methicillin resistance *S.aureus* show pus-filled lesions after 24 hours of infection with mean diameter (5.62 cm cm3) in all infected group, after 3and 7 days infected, non-treated group lesions reached maximum diameter (5.84 cm3) and (6.28 cm3) respectively, and when scarified animals, showed shin area with methicillin resistance *S.aureus* with severe ulcerative pyogenic lesion (figure 5,6). This results are in agreement with Godin *et al* (18) was observed animals infected with MRSA development of notable abscesses in the skin after 48hrs. Treated group with *aloe vera* AgNPs and clindamycin showed decrease in lesion volume with significant difference when compared with untreated group after 72h treatment, after 3 day *aloe vera* AgNPs significant reduction in lesion volume as compared with clindamycin and not treated groups, clindamycin showed less activity than *aloe vera* AgNPs in reducing the lesion volume when compared with untreated group (fig 7,8) and table (2). Reductions in lesion volume resulted in a decrease in *S.aureus* density in the skin.

Figure 5. Gross lesion of infected group after 24hrs. of pus-filled lesion

Figure 6. Gross lesion of infected Untreated group after 7day Post infection

Figure 8. Gross lesion of infected group after 3 days treated with clindamycin

Figure 7. Gross lesion of infected group after3 days treated with *aloe vera* AgNPs

Table 2. lesion size (cm3) of infected skin by *MRSA* treated with *aloe vera* AgNPs, clindamycin and non-treated group

Groups	48 hour after infection	3nd day of treatment	7 day of treatment
+ve control	5.12±0.25 A a	5.84±0.85 A a	6.28±0.13 A a
aloe vera AgNPs	5.28±0.42 A a	2.11±0.02 B b	0.00±0.00 C b
clindamycin	5.62±0.31 A a	4.12±0.24 B c	1.98±0.22 C c

Different capital letters mean significant (P<0.05) within groups
Different small letters mean significant (P<0.05) between group

The magnitude of the lesion volume score is generally in agreement with the magnitude of the decrease in colony forming unit. This result is in agreement with (32) who referred that treatment with clindamycin resulted in dose dose-proportional decreased in lesion volume for all doses ranging from 70% relative to untreated controls. Also (26) reported that inducible clindamycin resistance increased slightly from 20% to 25%, there was a prominent increase in constitutive clindamycin resistance from 0.1% to 26.8%. . These findings demonstrate a dramatic increase in clindamycin resistance among *S. aurous* isolates and suggest against the usage of clindamycin as empirical treatment for suspected *S. aurous* infections. Gel *aloe vera* extract was a good source for green synthesis stable AgNPs. Antibacterial activity of the synthesized AgNPs against gram positive and negative *in* vitro was dependent on the concentration of the AgNPs. Topical application of AgNPs accelerated healing of soft tissue infection by methicillin resistance *S. aureus*.

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