Biosynthesis of silver nanoparticles and evaluate its activity in promoting burns healing in rabbits

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Abstract

This study was conducted to synthesis silver nanoparticles by using olive leaves extract and evaluate its activity in promoting burns healing in rabbits. The synthesis and characterization of silver nanoparticles was confirmed by Ultra Violet Visible-spectrophotometer and Scanning Electron Microscopy. For this purpose, twenty five rabbits were used and divided randomly into equal five groups and subjected to second degree burn under general anesthesia by bar with brass circular end of 2.5cm diameter which immersed in boiling water (100°C) for 10 min and immediately it was placed on the rabbit back for 15 sec. without exerting any pressure just the weight of the instrument which was 350gm., these groups were: 1- Burns without any treatment and kept as negative control. 2- Burns treated with silver nanoparticles 3- Burns infected with *P. aeruginosa* and don't receive any treatment. 4-Burns infected with *P. aeruginosa* and treated with silver sulfadiazine and kept as positive control. 5-Burns infected with *P. aeruginosa* and treated with silver nanoparticles. Burn healing was assessed by measuring burn diameter at 7, 14, 21 and 28 days post burn treatment. The results of this study showed it possible to produce silver nanoparticles in ecofriendly and easy process and the results of burn healing demonstrated superiority of silver nanoparticles in reducing burn diameter at the end of the experiment. It could be concluded that olive leaves extract can be used effectively in the production of silver nanoparticles and these synthesized nanoparticles had positive effect in enhance burn healing when its applied locally.

Key words: Silver nanoparticles, Burn healing, Pseudomonas aeruginosa, rabbits
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التخليق الحيوي لدقائق الفضة النانوية ودراسة فعاليتها في تسريع التام الحروق في الارانب

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الخلاصة

أجريت هذه الدراسة لتخليق دقائق الفضة النانوية باستعمال أوراق الزيتون وتحديد فعاليتها في تسريع التام الحروق في الارانب. تم توصيف دقائق الفضة النانوية المخلقة حيوياً بواسطة جهاز المطياف الضوئي والمجهر الإلكتروني الماسح. استعمل في هذه التجربة خمس وعشرون ارنباً تراوحت اوزانها من 1400غم الى 2000 غم لدراسة تأثير دقائق الفضة النانوية في تسريع التام الحروق في جلد الارانب حيث قسمت هذه الحيوانات عشوائيا الى خمسة مجاميع متساوية بواقع خمس ارانب لكل واحدة منها وقد تم تعريضها جميعاً الى حرق من الدرجة الثانية في منطقة الظهر

وكانت هذه المجاميع على النحو الاتي: 1- حروق لم تتلق اي علاج واعتبرت سيطرة سالبة 2- حروق عولجت بدقائق الفضة النانوية 3- حروق خمجت بجرثومة الزائفة الزنجارية *Pseudomonas aeruginosa* ولم تتلق اي علاج 4- حروق خمجت بجرثومة الزائفة الزنجارية *Pseudomonas aeruginosa* وعولجت بالسلفر سلفا دايزين واعتبرت سيطرة موجبة 5- حروق خمجت بجرثومة الزائفة الزنجارية *Pseudomonas aeruginosa* وعولجت بدقائق الفضة النانوية، وقد كان العلاج بدقائق الفضة النانوية والسلفر سلفا دايزين موضعيا ويوميا وبتركيز 1% لمدة 14 يوماً. تم تقييم فعالية دقائق الفضة النانوية في العلاج من خلال قياس معدل قطر الحرق عند الايام 7 و14 و21 و 28 من تاريخ احداث الحرق. أظهرت نتائج هذه الدراسة أنه بالإمكان تخليق دقائق الفضة النانوية بطريقة سهلة وصديقة للبيئة، وقد كان لهذه الدقائق دور ملحوظاً في تسريع التام الحروق، فقد اظهرت النتائج تفوق المجاميع المعالجة بهذه الدقائق في تقليل قطر الحروق في نهاية التجربة مقارنة مع المجاميع الاخرى. يمكن أن نخلص إلى أن مستخلص أوراق الزيتون يمكن استعماله بشكل فعال في إنتاج دقائق الفضة النانوية، وكان لهذه الجسيمات النانوية تأثيراً ايجابياً في تسريع التام الحروق في الارانب عند استعمالها موضعيا.

الكلمات المفتاحية: لدقائق الفضة النانوية، التام الحروق، الزائفة الزنجارية، الارانب.

Introduction

Nanotechnology was predictable to open certain novel viewpoints to treat and prevent diseases by atomic measure skills of materials. Capability to discover the construction and role of bio-systems at the nano size, encourages investigation lead to progress in medicinal field (1). Nanoparticles (NPs) were gatherings of atoms in the scale of 1-100 nm. (2). Silver nanoparticles (AgNPs) were the most rapidly growing classes of nanoproducts (3). Silver has been used for many years in different fields of medicine as broad-spectrum antimicrobial agents (4), in cancer therapy (5) and in wound healing (6). The activity of AgNPs. was higher than silver ions because of the very small size and high surface area (7). Due to the environmental effects and energy consumption by using chemical and physical methods for AgNPs. production, the choice of biological method was most desirable, because this method was found to be not toxic and eco-friendly (8). Biogenic synthesis of AgNPs. by using plants and micro-organisms was beneficial because its decreases ecological effect in comparison with the other methods of synthesis, and it can be used to yield high amounts of NPs with distinct morphology and size (9, 10). Burn is one of the common medico-surgical problem all over the world. It is the most dangerous type of wounds, and, it imposes a serious burden on physical, mental, and socioeconomic conditions of the victim. Annually there were about 11 million people over the world suffered from burn injuries (11). For example In surveying study carried out to portray the numbers of burn cases in the city of Sulaymaniyah in Iraq, exhibited an aggregate of 947 burn cases were conceded throughout the year and the death rate was 28% (12). In Indian study, mortality as high as 40.3% among 2499 burn patients was reported (13). The utilization of topical treatment has been crucial and has enhanced the survival of patients with burns, and to reduce the occurrence of burn infection, which considered the main source of mortality in these patients. The present study was conducted to synthesis the AgNPs biologically by olive leaves extract and evaluate its role in promoting burn healing in rabbits.

Materials and Methods

Freshly leaves of olive collected from local olive trees in Baghdad. Then water was used to clean these leaves, and at that point let dry in room temperature. Then pounded to a fine powder by an electrical processor. Grouping the olive was conducted in the State Board for Seeds Testing and Certification S.B.S.T.C in Baghdad with document No. 1077 in 26/ 3/ 2014. The extract made by putting 50 gm. of prepared powder in 500 ml of sterile D.W. Then the mixture heated for 10 min. till the color of the mixture become faint yellow. At that point the obtained extract cooled to room temperature and separated (14). The synthesis of AgNPs was made by mixing 100 ml. of 10^3M AgNO3 solution with 5ml. of olive leaves extract with stirring to give a faint yellow solution at room temperature. The blend heated in a water bath at (40 and 60℃). The changing in color of the blend was monitored at different temperatures and times as explained here: 40℃ and 5 min of reaction time, 60℃ and 10 min. of reaction time and 60℃ and 15 min. of reaction time. The AgNPs obtained by centrifugation of the blend at 15.000 rpm for 10 min. then re-disposed in sterile D.W. to eliminate of any awkward materials (14). UV-vis. Spectrophotometer was used to study the optical features of biologically synthesized AgNPs. This apparatus used to confirmed Creation and constancy of AgNPs in sterile distilled water in wavelength ranged from 200 to 800 nm. The reduction of Ag+ to Ag° was checked by spectrophotometer after mixing the extract with AgNO3 solution, and the measuring was at regular intervals from 0 till 15 min. (15). The shape and size of synthesized AgNPs. was examined at advanced nano search center by using Scanning Electron Microscopy (SEM) as following: dropping very small amount of the sample on specialized grid (carbon coated copper) (16). Under general anesthesia by IM injection of xylazine 2% (V.M.D-Belgium) 4mg/kg BW and ketamine 10% (KEPRO-Netherland) 50 mg/kg BW (17), a twenty five rabbits were subjected to skin burn by bar with brass circular end of 2.5cm diameter (Fig. 1a), which immersed in boiling water (100°C) for 10 min and immediately it was positioned on the rabbit back for 15 sec. without exerting any pressure just the weight of the instrument which was 350 gm. (Fig. 1b). Then the rabbits divided into five equal groups as following: 1-Negative control: Burns without any treatment, 2-Burns treated locally daily with AgNPs at a concentration of 10mg/ml for 14 days (AgNPs group), 3-Burns inoculated with *P. aeruginosa* and did not receive any treatment (*P. aeruginosa* group), 4-Burns inoculated with *P.aeruginosa* and treated locally daily with silver sulfadiazine (SSD) at a concentration of 1% and kept as positive control (SSD + *P. aeruginosa* group) and 5-Burns inoculated with *P. aeruginosa* and treated locally daily with AgNPs at a concentration of 10 mg/ ml for 14 days (AgNPs+ *P. aeruginosa* group). The infection with *P. aeruginosa* by application 100µl containing 10^7CFU of total bacteria at the site of burn immediately after burn induction.

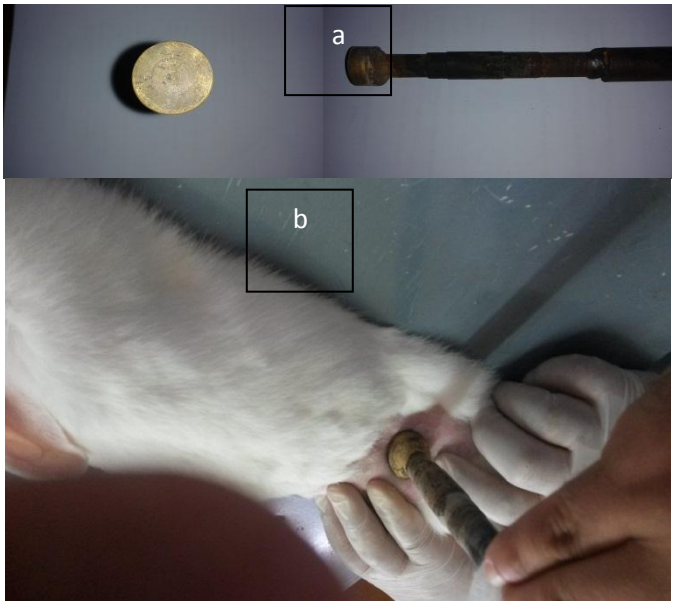

Fig. (1) a- Instrument used for burn induction. b-Application of the instrument on the back of the rabbit without any pressure just the weight of the instrument.

Diameter of induced burn was measured using a caliper. The measure was performed as procedure described by (18), in which the diameter was measured four times as can be seen in (Fig. 2), then the average from four measurements was calculated. The burn wounds diameter was measured immediately after burn induction and then at 7, 14, 21 and 28 days post burn treatment.

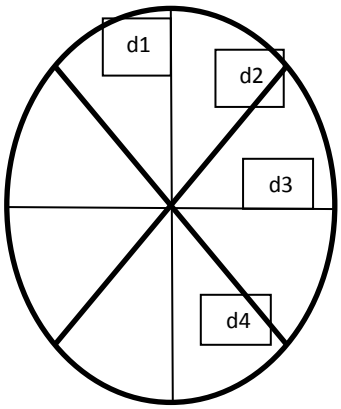

Fig. (2) Method of measuring the diameter of burn wound (18).

Measurements was calculated: dx= **(d1 + d2 + d3 + d4)/4**

Note: dx is the burn diameter on day x; d1=diameter 1; d2=diameter 2 etc.

Results and Discussion

The current study provided evidence that the olive leaves were great source for synthesizing stable AgNPs in lesser time. When olive leaves extract was mixed with the AgNO3 solution at room temperature gave pale yellow color, after 5min. of reaction time at 40℃ the color of the solution altered from faint yellow to profound or deep yellow color at 40℃ and 5 min. of reaction time. This change in color indicating formation of AgNPs. due to reduction of Ag+. When the temperature of water bath increased to 60℃ for 10 min., the color of blend altered to deep brown color, and finally the color of the mixture became grey-black at 60℃ after 15 min. of reaction time (Fig. 3).

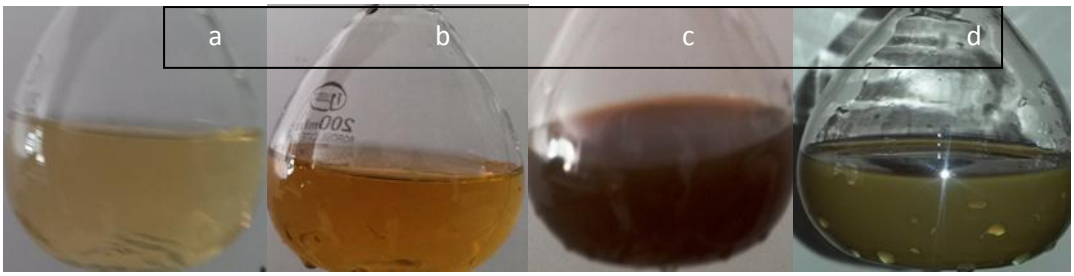

Fig. (3) Color change during synthesis of silver nanoparticles a- Pale yellow color when olive leaves extract was mixed with the silver nitrate solution. b- Deep yellow color at 40℃ after 5 min. of reaction time. c- Deep brown color at 60℃ after 10 min. of reaction time. d- Grey-black color at 60℃ after 15 min of reaction time.

The mechanism by which the plant extract could be synthesized AgNPs may be explained by the higher total content of phenols and flavonoids (19, 20). These phenols and flavonoids have high reducing capacity which lead to formation of the AgNPs (21, 22). These NPs. exhibited yellowish brown color in aqueous solution due to excitation of surface plasmon vibrations in AgNPs, and this result has been previously obtained by several investigators (23 , 24, 25). Increasing the temperature of water bath to 60℃ and 10 min. of reaction time, the color of mixture changed to deep brown color and at 15 min. of reaction time at 60℃ the deep brown color changed to grey-black, this further color change was due to increased concentration of NPs. with assisting of temperature (26). The resulted color of mixing olive leaves extract with AgNO3 solution was pale or faint yellow color, examination the mixture at this time which considered zero time by Uv-vis spectra revealed no sign and no peak for the synthesis of AgNPs. At 40℃ after 5 min. of the reaction time, the color altered from faint yellow to deep yellow because of surface plasmon vibrations of the AgNPs. became excited which produced a peak centered at 420nm. which corresponds to the absorbance of AgNPs. Also at 60℃ after 10 min. of the reaction time and 60℃ after 15min. of the reaction time the peak of the absorbance at 430nm. and 420nm. respectively (Fig. 4). This result agreed with several studies showed that the AgNPs surface plasmon vibrations peak at around 420 nm. (24, 27, 28).

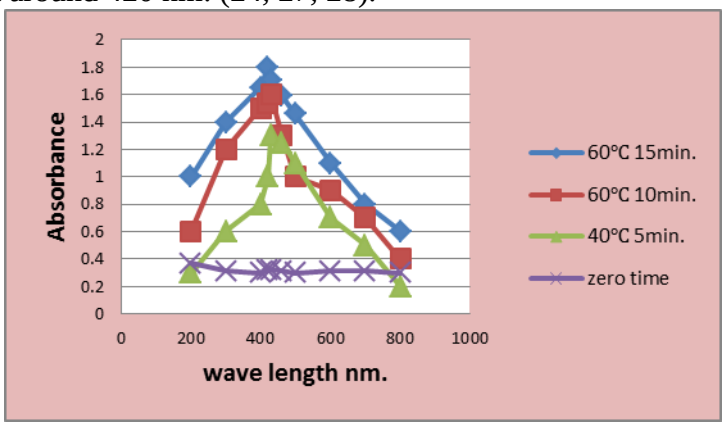

Fig. (4) UV-Vis absorption spectra of AgNPs synthesized by olive leaves extract at different times and temperatures.

NPs. have optical properties that were sensitive to size, shape, concentration and agglomeration state and strongly interact with specific wavelengths of light. This strong interaction with light occurred because the conduction electrons on the metal surface undergo a collective oscillation when they were excited by light at specific wavelengths and this oscillation is known as a surface plasmon resonance (SPR), and it causes the

absorption and scattering intensities of AgNPs to be much higher than identically sized non-plasmonic NPs., so at zero time, Uv-vis. spectra showed no peak because the AgNPs not formed yet i.e. there was no any concentration of AgNPs, whereas at 5, 10 and 15 min. after reaction, there was increase in absorbance due to increase NPs. concentration and the Uv-vis. spectra showed peak at 420nm. and 430 nm, which corresponds to the absorbance of AgNPs (29). These peaks appeared single and narrow which mean the biologically synthesized AgNPs. were stable and didn't aggregated, which may be due to AgNPs. had negative surface charge and this high negative charge of particles increases its stability (more than 1 year) due to repulsion between the particles that avoid its aggregation (30). SEM gave further understanding into the size and shape details of the synthesized AgNPs. The outcomes demonstrated that the mean diameter of produced AgNPs. was around 76 nm. Also the image showed relatively spherical shaped NPs. (Fig. 5).

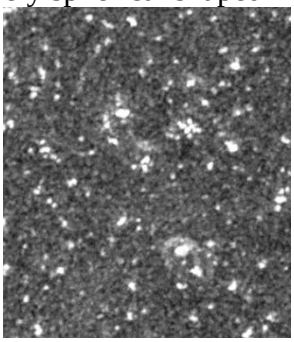

Fig. (5) SEM image showed synthesis of AgNPs with mean diameter 76nm. with relatively spherical

Therapeutic effect was assessed on the physical appearance and reduction of wound diameter**.** Immediately after burn induction the burned skin showed paleness in all experimental animals (Fig. 6). This paleness in color may be due to denaturing of the proteins within the skin. Coagulation is the area of close contact with the heat source. Absence of blood flow to the area produced necrosis which consists of dead cells. So, as a result of coagulative necrosis and absent blood flow the affected area usually appears pale.

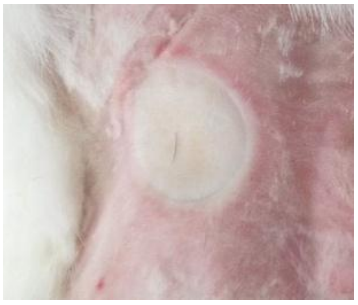

Fig. (6) Gross appearance of second-degree burn which appear pale in color

The burn in group inoculated with *P. aeruginosa* which did not receive any treatment the inflammation changed to infected burn which lead to more severe signs of inflammation included redness, swelling and warmth. Then after three days presence of pus on the burned area confirmed the infection which persisted along the period of study. These signs (redness, swelling and warmth) induced by burn seemed to be less in negative control groups and almost absent or slight in AgNPs, AgNPs+ *P. aeruginosa* and SSD+ *P. aeruginosa* groups. At the day 7 post burn, all the burn wounds except *P. aeruginosa* group were covered with scab which sloughed after day 21 in control and SSD+ *P. aeruginosa* groups and after day 14 in AgNPs and AgNPs+ *P. aeruginosa* groups, while its was covered with blood and pus in the *P. aeruginosa* group (Fig. 7).

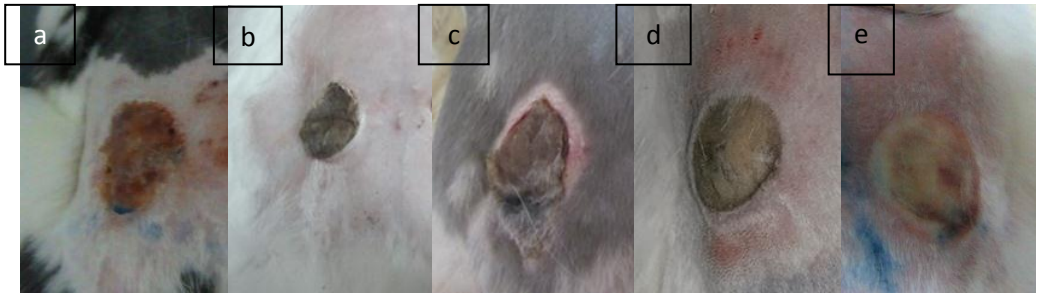

Fig. (7) Burned skin of rabbits at day 7 post burn. a- Control group: the burn covered with scab with inflammation the burned area and burn diameter mean was 2.2cm. b- Group treated with AgNPs: the burn covered with scab and no noticeable signs of inflammation with diameter mean 1.7cm. c- Group infected with *P.aeruginosa* and treated with AgNPs: the burn covered with scab with no evidence of inflammation and burn diameter mean was 1.9cm. d- Group treated with SSD: the burn covered with scab slight signs of inflammation and burn diameter mean was 1.9cm. e-Group infected with *P.aeruginosa* without any treatment: burned skin filled with pus and spreading the inflammation to the surrounding area and burn diameter mean was 2.5cm.

At day 14 post burn there was improvement in burned skin by formation thin epithelial layer under the scab in AgNPs, AgNPs+ *P. aeruginosa* and SSD+ *P. aeruginosa* groups as compared with control group and *P. aeruginosa* group which showed remaining the inflammation and pus in the burn (Fig. 8).

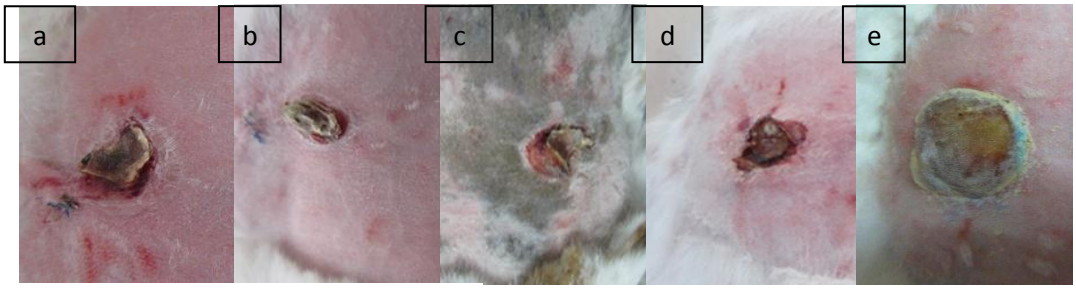

Fig. (8) Burned skin of rabbits at day 14 post burn. a-Control group: the burn remained covered with scab and did not show evidence of healing and burn diameter mean was 2cm. b-Group treated with AgNPs: the burn covered with scab and can be seen formation thin epithelial layer under the scab and burn diameter mean was 1cm. c-Group infected with *P.aeruginosa* and treated with AgNPs: there was formation of epithelial layer under the scab and burn diameter mean was1.2 cm. d- Group treated with SSD: the burn still covered with scab with develop thin epithelial layer and burn diameter mean was 1.4 cm. e-Group infected with *P.aeruginosa* without any treatment: persistence of inflammation and pus in the burned skin and burn diameter mean was 2.5 cm.

At day 21 after burn induction was slight improvement in burned skin of control group by formation thin layer of epithelium under the scab, and there was progression in epithelial layer formation in AgNPs, AgNPs+ *P. aeruginosa* and SSD+ *P. aeruginosa* with very slight inflammation in the last group (SSD) while no observed improvement in *P. aeruginosa* group (9).

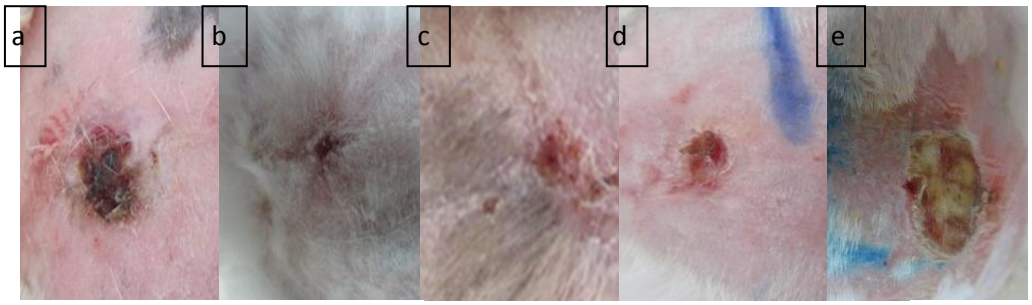

Fig. (9) Burned skin of rabbits at day 21 post burn. a- Control group: the burn still covered with scab and there was slight improvement in healing by formation thin epithelial layer and burn diameter mean was 1.8cm. b- Group treated with AgNPs: the scab was sloughed with developing in epithelial layer formation and diameter mean was 0.5 cm. c-Group infected with *P.aeruginosa* and treated with AgNPs: there was detachment of the scab with progression in epithelium and burn diameter mean was 0.7cm. d- Group treated with SSD: the burn remained covered with scab with develop epithelial layer and burn diameter mean was 0.8 cm. e-Group infected with *P. aeruginosa* without any treatment: the burn showed severe signs of inflammation and burn diameter mean was 2.6 cm.

At day 28 post burn, both AgNPs and AgNPs+ *P. aeruginosa* groups revealed better signs of healing, where the epithelial layer fully formed with growing of hair and with lesser extent in SSD+ *P. aeruginosa* group in comparison with control group which showed just thin epithelial layer and *P. aeruginosa* group which not showed any signs of healing in contrast there was slight increase in burn size (Fig. 10).

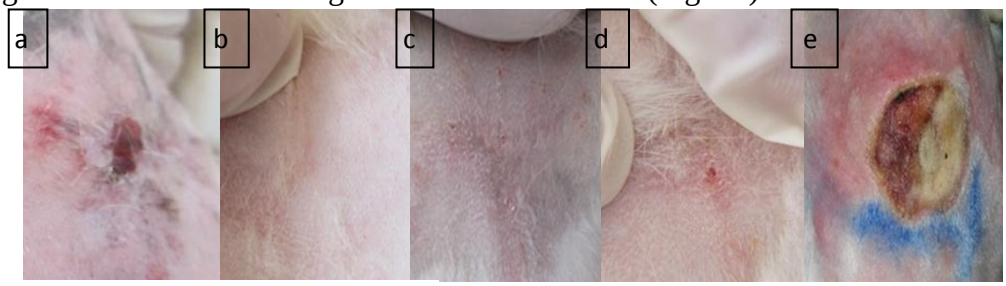

Fig. (10) Burned skin of rabbits at day 28 post burn. **a-** Control group: the scab was sloughed with moderate healing and burn diameter mean was1.4cm. **b-** Group treated with AgNPs: revealed better signs of healing, where the epithelial layer fully formed with growing of hair and burn diameter mean was 0.08cm. **c-** Group infected with *P.aeruginosa* and treated with AgNPs: showed curative effect in epithelium with hair growing and burn diameter mean was o.1cm. **d-** Group treated with SSD: there was detachment of the scab with nearly complete formation of epithelial layer and burn diameter mean was 0.4 cm. **e-**Group infected with *P.aeruginosa* without any treatment: the burn showed poor healing by continuous sever signs of inflammation with presence of pus and burn diameter mean was 2.7cm.

The diameter of the burn on the first day was considered as 100% (2.5cm.) and burn diameter on subsequent days (7, 14, 21 and 28) were compared with the burn diameter on the initial day. The comparison between periods (7, 14, 21, and 28 days) of burn diameter measurement after burn induction of the same group at ($P<0.05$) showed significant decrease in burn diameter between periods of all groups except periods of burn diameter measurement of *P.aeruginosa* group, which exhibited significant increase in burn diameter at day 28 post burn as compared to day 14 and day 7 post burn while the differences were not significant between other three periods table (1). The findings of the comparison between burns diameters measurements at day 7 post burn demonstrated that the group treated with AgNPs., showed superiority in reduction burn diameter (1.70±0.50 cm.) with statistical difference at ($P<0.05$) in comparison with the other groups where the mean of burn diameters were (2.20±0.13, 1.90±0.60, 1.90±0.30, 2.50±1.00) cm. in control, SSD+ *P.aeruginosa*, AgNPs + *P.aeruginosa* and *P.aeruginosa* respectively. There was no statistical difference in burn diameter between SSD+ *P.aeruginosa* and AgNPs + *P. aeruginosa* at ($P<0.05$) but they significantly less than of these in control and *P.aeruginosa*

groups, which also there was statistical difference between them (control and *P.aeruginosa*) at (P<0.05) table 15 and Fig. 7. At day 14 post burn the findings demonstrated there were statistical differences at (P<0.05) between all groups in comparison between them with excellence of AgNPs. group where the means of burn diameters were (2.00±0.78, 1.00±0.73, 1.40±0.84, 1.20±0.80 and 2.50±0.1.40) cm. in control, AgNPs., SSD+ *P. aeruginosa*, AgNPs + *P. aeruginosa* and *P. aeruginosa* respectively table 1 and Fig. 8. At day 21 post burn the results revealed topping the AgNPs. group with significant difference in reduction burn diameter (0.50±0.55 cm.) at (P<0.05) as compared to other groups where the burn diameters were 1.80±0.60 cm. in control, 0.80±0.72 cm. in SSD+ *P. aeruginosa*, 0.70±0.83 cm. in AgNPs + *P. aeruginosa* and 2.60±0.96 cm. in *P.aeruginosa*, and there were statistical differences between these groups at (P<0.05) except between SSD+ *P.aeruginosa* and AgNPs + *P.aeruginosa* groups there was variance in diameter reduction but not significant table 1 and Fig. 9. At day 28 post burn the burns diameters measurements showed superiority of AgNPs. and AgNPs.+ *P. aeruginosa* groups in reduction burn diameter with significant difference at (P<0.05) with respect to other three groups which showed significant differences between them, where the burns diameters were (1.40±0.68, 0.08±0.70, 0.40±0.67, 0.10±0.75 and 0.70±1.20) cm. in control, AgNPs., SSD+ *P. aeruginosa*, AgNPs + *P. aeruginosa* and *P. aeruginosa* respectively table (1) and Fig. (10).

Table (1) Mean of burn diameter (cm.) in rabbits after 7, 14, 21 and 28 days post burn

Periods (Days) / Groups	7	14	21	28
Control	2.20±0.13 Ab	2.00±0.78 Bb	1.80±0.60 Cb	1.40±0.68 Db
AgNPs	1.70±0.50 Ad	1.00±0.73 Be	0.50±0.55 Cd	0.08±0.70 Dd
SSD + *P.aeruginosa*	1.90±0.60 Ac	1.40±0.84 Bc	0.80±0.72 Cc	0.40±0.67 Dc
AgNPs+ *P.aeruginosa*	1.90±0.30 Ac	1.20±0.80 Bd	0.70±0.83 Cc	0.10±0.75 Dd
P.aeruginosa	2.50±1.00 Ba	2.50±0.1.40 Ba	2.60±0.96 BAa	2.70±1.20 Aa

The different capital letters refer to a significant differences between rows at (P<0.05).
The different small letters refer to a significant differences between columns at (P<0.05).
Values represent Mean ± SE.

The result of burn healing was in agreement with other studies showed that the application of AgNPs. enhanced wound healing (31, 32, 33, 34). AgNPs. could improve the healing of burn initially on the basis of the known antimicrobial property, as well as the anti-inflammatory effect. A great problem in the burn healing is the bacterial contamination. The presence of bacteria in the burn delays the burn-healing process because of competing with host cells for nutrients and oxygen. Furthermore, their waste products are also toxic to host cells (35). These reasons might be explain why the group infected with *P. aeruginosa* and didn't receive any treatment showed poor signs of healing with progressed inflammation along the period of study. In this study, groups that treated with AgNPs showed promoting in burn healing with no or slight signs of inflammation because the AgNPs played a role in altering or compressing the inflammatory events in burns and facilitating the early phases of burns healing. The inflammatory response is an important component of burn healing. To accomplish successful burn repair and tissue regeneration, the inflammatory response must be tightly regulated in vivo. Excessive

inflammation, this kind of inflammation is not a response to infection, and is known as aseptic inflammation. is represent therapeutic challenge in burns and chronic wounds (36). AgNPs increases the level of the IL-10, a vital mediator in this anti-inflammatory cascade. One of the unique actions of IL-10 is its ability to inhibit the synthesis of pro-inflammatory cytokines, like IL-6. Furthermore, silver was proven to have other beneficial effects on the burn bed. A number of the biochemical effects of silver on the burn have been documented, there was important relationship between tissue destruction by a group of collagenase enzymes known as MMP and tissue synthesis which is stimulated by growth factors. It is well recognized that MMP were needed to heal a wound, but excess levels degrade fibronectin and peptide growth factors. This effect is exacerbated further by diminished levels of tissue inhibitors of metalloproteinase (TIMPs) (37). Silver-based technologies in particular provide added benefits by down-regulating MMPs to levels that facilitate wound healing (38). In conclusion, the olive leaves extract were good source for synthesis of stable AgNPs. in short time, with simple, low cost and eco-friendly method, and both temperature and time played an important role in accelerating the synthesis of AgNPs. We concluded also the AgNPs. had positive effect in enhance burn healing when its applied locally.

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