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ORIGINAL ARTICLE

Formulation and characterization of a novel cutaneous wound healing ointment by silver nanoparticles containing *Citrus lemon* leaf: A chemobiological study

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KEYWORDS

Citrus lemon leaf aqueous extract;
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Abstract *Introduction:* Formulating new wound-healing ointments by natural compounds is the first research priority in the developing and developed countries. This study was intended to provide green formulation of Ag-NP ointment containing Citrus lemon leaf aqueous extract and examine its capability of healing cutaneous wounds and its antioxidant and cytotoxicity activities under in vitro and in vivo conditions.

Materials and methods: Different techniques, including UV–Vis and FT-IR spectroscopy, were used to characterize Ag-NPs. MTT assay was used to investigate cytotoxicity property of Ag-NPs. Antioxidant activity of Ag-NPs were examined by DPPH in the presence of butylated hydroxytoluene as positive control. Parameters of cutaneous wound healing were measured both histopathologically and biochemically.

Results: Clear peak at 429 nm shown by UV–Vis spectroscopy indicated formation of Ag-NPs. In FT-IR spectroscopy, presence of many antioxidant compounds provided an excellent condition to reduce silver in Ag-NPs. FE-SEM and TEM images showed spherical Ag-NPs with an average size of 25.1 nm. The synthesized silver nanoparticles had excellent cell viability on the HUVECs line and indicated this method was nontoxic. Application of Ag-NP ointment improved wound healing

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parameters significantly ($P \leq 0.01$). Ag-NPs reduced wound areas, total cells, neutrophils and lymphocytes significantly ($P \leq 0.01$) and increased wound contracture, vessels, hexosamines, hydroxyl proline, hexuronic acid, fibrocytes, fibroblasts and fibrocyte/ fibroblast ratios significantly ($P \leq 0.01$).

Conclusions: Once our results are verified by clinically experimental studies, Ag-NP ointment can be used as a modern one to treat several types of wounds, especially cutaneous ones, in humans.

1. Introduction

Nanotechnology is a rapidly developing part of science involving the synthesis and evolution of various nanomaterials. At present, Ag nanoparticles have not only strong catalytic ability, but also bactericidal properties and antioxidant capacity, which are widely used in medical and health care, food packaging, drinking water disinfection, personal life care, and other fields (Zangeneh, 2020; Mohammadi et al., 2020; Hamelian et al., 2020; Hemmati et al., 2019; Jalalvand et al., 2019). Ag nanoparticles are quite different from their bulk materials in terms of optical, chemical, electrical and biological properties (Zhaleh et al., 2019; Shahriari et al., 2019; Goorani et al., 2020). Ag nanoparticles synthesized by conventional chemical and physical methods are expensive and may be harmful to the environment. Therefore, it is very important to find a clean and environment-friendly method to prepare Ag nanoparticles. Biologically green method of synthesizing metal oxide NPs by medicinal plants is a single-step, bio-reduction, eco-friendly and cost-effective method requiring comparatively lower amounts of energy to initiate reaction processes. The most favourable method of Ag-NPs synthesis is green one since it creates safe, clean and homogenous nanoparticles by a simple and reasonable technique during maintenance of biological contribution (Zangeneh, 2020; Mohammadi et al., 2020; Hamelian et al., 2020; Hemmati et al., 2019;). Nanoparticles with phytochemicals surrounding Ag-NPs surfaces are functionalized biologically by green method using plant extracts. Not only interactions among nanoparticles, but also responses by cutaneous wound healing, hyperglycemic, anticancer and bacterial cells to the target actions are restricted by such bio-functionalization of NPs. As indicated by previous studies, Ag-NPs green-formulated by medicinal plants exhibited significant cutaneous wound healing activities under in vitro and in vivo conditions. As the application of Ag-NP-based ointment improved parameters of cutaneous wound healing, it can be said that such NPs are able to decrease wound area and numbers of total cells, neutrophils and lymphocytes while increasing levels of wound shrinkage, vessels, hexosamines, hydroxyl proline, hexuronic acid, fibrocytes, fibroblasts, and fibrocytes/fibroblasts compared to other groups (Jalalvand et al., 2019).

Silver nanoparticles have great potential in detecting, drug delivery, coating biological materials. These nanoparticles also have antibacterial properties. These properties have led to many applications of silver nanoparticles in medicine, including wound healing.

Science history is an enticing field of humanity's interdisciplinary knowledge and study. Medical science is one of the interesting fields in science history. Iran, as an ancient civilized nation, has a long medical history with the best-known people involved (Zangeneh, 2020; Mohammadi et al., 2020; Hamelian et al., 2020; Hemmati et al., 2019). Traditional Iranian medicine is one of the most famous herbal medicines, developing various medicinal supplements and medications used for the diagnosis, control and prevention of many diseases yearly (Zangeneh, 2020; Mohammadi et al., 2020; Hamelian et al., 2020; Zhaleh et al., 2019; Shahriari et al., 2019; Goorani et al., 2020). Anticancer properties of traditional Iranian medications have been used by Iranian researchers to synthesize and develop several chemotherapeutic supplements and drugs, one of which is Citrus lemon (Zangeneh, 2020). The recent species is rich of antioxidant molecules including α-Pinene, Sabinene, β-Pinene, Δ-3-Carene, α-Terpinene, p-Cymene, Limonene, *cis*-Ocimene, γ-Terpinene, Linalool oxide, α-Terpinolene, Linalool, *cis*-p-Menth-2-en-1-ol, Terpinen-4-ol, α-Terpineol, Linalyl acetate, Geraniol, α-Terpinyl acetate, Piperitenone oxide, Neryl acetate, Geranyl acetate, β-Elemene, trans- Caryophyllene, β-Farnesene, α-Humulene, Germacrene D and α-Farnesene (Al-Jabri et al., 2014; Di Vaio et al.;2010; Espina et al., 2011; Golmakani and Moayyedi, 2015). The seeds and leaves of *Citrus lemon* and their products such as seeds tincture and roasted black seeds have been used in different regions of the world to treat diseases such as rheumatism, bronchitis, asthma, rheumatism, dropsy, loss of appetite, treatment of indigestion, diarrhea, dysmenorrhoea, amenorrhoea, skin eruptions, worms and vomit (Al-Jabri et al., 2014; Di Vaio et al., 2010; Espina et al., 2011; Golmakani and Moayyedi, 2015; Ifesan et al., 2013). In Iranian traditional medicine, people use the leaves of *Citrus lemon* to treat cutaneous wounds. Probably, therapeutic properties of recent species are associated with its antioxidant compounds (Golmakani and Moayyedi, 2015).

Objectives of this study were to prepare green Ag-NP ointment formulation using Citrus lemon aqueous leaf extract and explore its cutaneous wound healing, antioxidant and cytotoxicity properties under in vitro and in vivo conditions. Due to high antioxidant effects of Ag-NPs green-synthesized by Citrus lemon leaf extract, we assumed that they would show excellent wound-healing efficacy during the use of histopathological and biochemical approaches in vivo.

2. Materials and methods

2.1. Materials

Bovine serum, antimycotic antibiotic solution, 2,2-diphenyl-1-pikrilhydrazil (DPPH), dimethyl sulfoxide (DMSO), decamplmaneh fetal, 4- (Dimethylamino) benzaldehyde, hydrolyzate, Ehrlich solution, and borax-sulfuric acid mixture, Dulbazolic mixture Modified Eagle Medium (DMED) all were obtained from the US Sigma-Aldrich company.

2.2. Synthesis of Ag nanoparticles containing Citrus lemon leaf aqueous extract

Extraction from Citrus lemon leaf is a technique used for preparing green-synthesized Ag-NPs as the first step. To achieve this end, we extracted from 25 g of Citrus lemon leaves by using distilled water in a microwave.

Generally, this procedure was performed as follows: 1.5 g of NaOH pellets plus 100 ml of $AgNO_3$ were dissolved in 50 ml of distilled water [(Merck, CAS# 7761–88-8) × H_2O (5×10^{-4}M)], to which 20 ml of Citrus lemon leaf extract were added and stirred at 25 °C for 1 h. After this process, the solution color changed into black, indicating the formation of Ag nanoparticles. Finally, the solution was let to precipitate, then, the precipitations were filtered and washed with ethanol, acetone, and distilled water. Final precipitations were dried at 90 °C for 14 to provide the best Ag nanoparticles powder (Zangeneh, 2020; Mohammadi et al., 2020).

2.3. Chemical characterization of Ag nanoparticles containing Citrus lemon aqueous extract

Several techniques were used to characterize nanoparticles, including TEM, UV–Vis and FT-IR (Zangeneh, 2020).

TEM is a method by which the size and shape of nanoparticles were determined (Zangeneh, 2020). UV–Vis spectroscopy analysis is a method by which the characteristic absorption bands of silver were evaluated (Zangeneh, 2020).

FT-IR (Shimadzu IR affinity.1) was used to identify the potential biomolecules in the *Citrus lemon* aqueous extract that participated in the reduction of silver nanoparticles (Zangeneh, 2020).

2.4. Antioxidant activities of Ag nanoparticles containing Citrus lemon aqueous extract

2, 2- diphenyl-1-picrylhydrazyl (DPPH) was used to examine antioxidant activities of Ag-NPs, Citrus lemon aqueous leaf extract and silver salts (Hosseinimehr et al., 2011). First of all, 100 ml of CH_3OH were added to 39.4 g of DPPH to prepare its solution which was added to different concentrations of Ag-NPs, Citrus lemon aqueous leaf extract and silver salt (0–1000 µg/mL). Then we added DPPH solution to samples, as explained above, and incubated it at 37 °C for 30 min. After that, absorbances of the mixture were determined at 570 nm. In this experiment, CH_3OH (50%) and butylated hydroxytoluene (BHT) were considered negative and positive controls. Antioxidant activities of samples were calculated according to the following formula:

$$\text{Inhibition}(\%) = \frac{\text{Sample A.}}{\text{Control} - \text{A.}} \times 100$$

2.5. Cytotoxicity properties of Ag nanoparticles containing Citrus lemon aqueous extract

In this research, we used Human Umbilical Vein Endothelial Cells (HUVECs) to evaluate cytotoxicity effects of Ag nanoparticles, *Citrus lemon* leaf aqueous extract and silver salt using MTT.

These cell lines were plated on DMEM media using 96-well plates with antimycotic solution, 10% FBS, streptomycin and penicillin. The number of cells in each plate-based well was 10,000, which were incubated at 37 °C for 24 h and treated with Ag nanoparticles, *Citrus lemon* leaf aqueous extract and silver salt with different dilation sizes, (0–1000 µg/mL) and incubated for 24 h. Then, 5 mg/ml of MTT were added to all wells, which were finally incubated at 37 °C for 4 h. After the evaluation of absorbance at 531 nm, the percentage of cell viability was calculated from the following equation (Arulmozhi et al., 2013):

$$\text{Cell viability}(\%) = \frac{\text{Sample A.}}{Control\ A.} \times 100$$

2.6. Cutaneous wound healing

All procedures to be performed on the test animals were approved by Research Ethics Committee of Health and Medical Education Ministry of Iran through Standards based on Helsinki Protocol (Helsinki, Finland, 1975) adopted by Payam-e Noor University of Kermanshah (No. 01/Z/G 1395/12/01) in April 17, 2006.

To perform experiments, 60 male, 10-week-old Sprague Dawley rats weighing 210–220 g were used. Under conditions of ambient temperature of 21 °C and 50–60% humidity, the rats were kept separately in transparent plastic cages placed in a noise-free room with dim lighting in 12-hour light/dark cycles. The rats had non-restricted access to standard water and food pellets during experiments.

A wound (2×2 cm) involving the removal of all skin layers was created by a scalpel after the induction of anesthesia by intramuscular injection of 40 mg/kg Ketamine, depth of which contained dermis and hypodermis (Fig. 1). Then, the rats were assigned randomly to 6 groups (n = 10) as follows: treated with (1) 0.2% Ag-NPs ointment; (2) 0.2% Ag salt ointment; (3) 0.2% Citrus lemon ointment; (4) 3% tetracycline ointment; (5) Eucerin basal ointment; and (6) untreated (control) groups. In this study, 0.2% Ag-NP and Citrus lemon ointments were formulated by using Eucerin basal ointment.

The ointment has been applied to the wound beds for the next 10 days. By monitoring the wounds using transparent paper and a permanent marker, percentages of wound contracture were evaluated on day 10. Some graph paper with 1 mm^2 area was used to measure recorded wound areas, the changes of which were assessed to obtain an indication of duration of wound contraction period of time. Taking the initial wound size as 100%, percentages of wound contracture were calculated using the measured surface areas, as shown below:

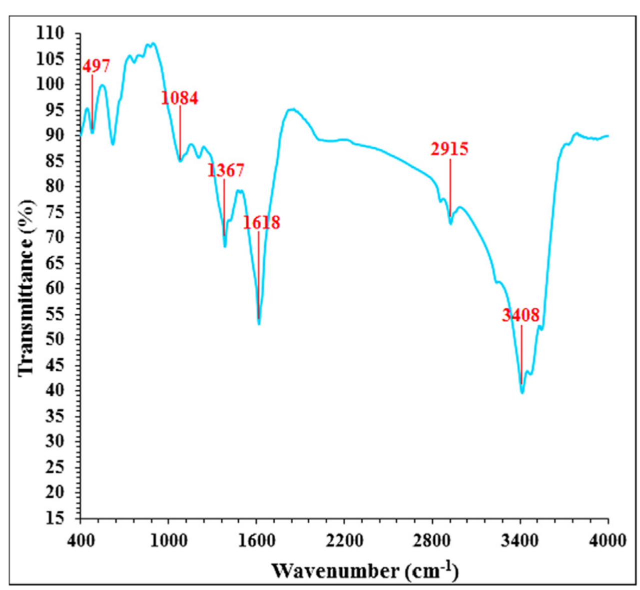

Fig. 1 FT-IR spectra of silver nanoparticles green-synthesized by *Citrus lemon* leaf.

$$\% \textit{ Wound contracture} = (\textit{Wound area on } 1st \textit{ day} - \textit{Wound area on } 10th \textit{ day})/\textit{Wound area on } 1st \textit{ day} \times 100$$

Wound samples were taken from each group after 10 days of treatments. Equally split histological sections were placed in 10% formalin and sent to the lab. One half of each sample was examined histologically by 2 pathologists performing a slightly modified procedure reported (Ghashghaii et al., 2017; Zangeneh et al, 2019). Then, photos were captured by a digital camera (Dino capture; version 1.2.7) and entered into computer software (Photoshop C2-4; Adobe) to be analyzed digitally. Histopathological analyses were performed using 5 photomicrographs selected from 5 microscopic fields of each tissue sample. Parameters evaluated using histopathological sections included fibrin deposition, hemorrhage, polymorph nuclear and mononuclear cell infiltration, re-epithelialization, epithelium cornification, re-vascularization, fibroblast and macrophage contents, necrosis, presence of fibrocytes, and collagen maturation and organization. Once total cellularity (magnification × 200) and numbers of fibroblasts, fibrocytes, lymphocytes, neutrophils and blood vessels (magnification × 800) of wound area have been counted, their means (M) and standard deviations (SD) were calculated. Other half of each sample underwent biochemical studies to determine concentrations of hexosamine, hexuronic acid and hydroxyl proline (Ghashghaii et al., 2017).

2.6.1. Hexosamine measurement

Initially, 0.5 ml of acetyl acetone was added to Eppendorf of cutaneous wound samples. Then, Eppendorf tubes were heated in 75 °C bain-marie for 20 min, and, next, they were placed and kept in cold water for 5 min. Within 30 min after the addition of 1.5 ml of 95% alcohol and 0.5 ml Ehrlich solution to samples, hexosamin concentration was measured at 530 nm wavelength (Ghashghaii et al., 2017).

2.6.2. Hexosamine acid measurement

2.5 ml of 0.025 M Borax on concentrated sulphuric acid were added to stoppered tubes fixed in a rack, and cooled to 4 °C. Addition of distilled water led to the dilution of 0.125 ml hydrolysate to 0.58 ml, which was now layered carefully on Borax-suplhuric acid mixture kept in a rack at 4 °C. Using glass stoppers, tubes were closed and shaken first slowly and then vigorously. Then, they were placed in an ice container to be cooled constantly. After that, tubes were heated in an over-boiling water bath for 10 min and, then, cooled to the room temperature. Next, 0.1 ml 0.125% carbazole reagent in absolute alcohol was added to each tube, shaken and reheated in the boiling water bath for more 15 min, then, tubes were cooled to the room temperature. Measurement of color intensity was done at 530 nm against the blank. The standard curve prepared using D (+) Glucurono-6, 3- lactone was employed to determine the content of hexuronic acid in each sample (Ghashghaii et al., 2017).

2.6.3. Hydroxyproline measurement

First, samples were transferred to 5 ml Eppendorf to which 0.3 ml hydrolysate, 2.5 ml of N NaOH, 0.01 M $CuSO_4$, and 6& H_2O_2 were added. Next, Eppendorf tubes were transferred to 80 °C bain-marie and cooled in cold water for 5 mm within 15 min after being transferred. Then, 0.6 ml 5% paradimethyl amino-benzaldehyde and 1.2 ml 3 NH_2SO_4 were added to Eppendorf. After that, Eppendorf tubes were placed in 75 °C bain-marie for 15 min and, then, transferred to and kept in cold water for 5 min. Ultimately, a spectrophotometer was used to measure hydroxyproline concentration at 540 nm wavelength (Ghashghaii et al., 2017).

2.7. Statistical analysis

Data was gathered and entered into "SPSS-24", a computer software program, and analyzed by "one-way ANOVA" and then by Duncan post hoc test ($p \leq 0.01$).

3. Results and discussion

Because of possessing valuable secondary metabolites and many pharmaceutical properties, medicinal plants have attracted researchers' attention and interest (Zangeneh, 2020; Mohammadi et al., 2020; Hamelian et al., 2020; Shahriari et al., 2019; Goorani et al., 2020). In recent years, several studies were carried out on different aspects of plants. An extensively studied aspect of medicinal plants is their curing effects versus chemical medications (Zangeneh, 2020; Mohammadi et al., 2020; Hamelian et al., 2020). Another aspect is the use of such plants to produce nanoparticles instead of using chemical methods because the latter are slow, expensive and harmful for both humans and environment. Known as green chemistry, this plant-centered method of producing nanoparticles has been paid to much more attention recently (Zhaleh et al., 2019; Shahriari et al., 2019; Goorani et al., 2020). Previous studies demonstrated stronger treatment effects for nanoparticles produced by plants compared to plants alone (Zangeneh, 2020; Shahriari et al., 2019; Goorani et al., 2020). Our study was intended to assess effects of Ag-NPs containing Citrus lemon aqueous extract on cutaneous wound healing. Results showed that new NPs had significant antioxidant and cutaneous wound healing properties free from any cytotoxic effects against human cell lines (HUVEC).

3.1. Chemical characterization of Ag nanoparticles containing Citrus lemon aqueous extract

As a mathematical technique, Fourier transform infrared spectroscopy (FTIR) is used to obtain infrared spectra of absorption or emission of a liquid, solid or gas. FTIR spectrometer collects high resolution spectral data over a wide spectral range simultaneously. This can be considered a significant advantage of FTIR spectrometer over the dispersive one measuring intensity merely over a narrow wavelength range at a time. FTIR technique is an adequate way to detect bioactive components in the field of naturally occurring products (Zangeneh, 2020; Mohammadi et al., 2020; Hamelian et al., 2020; Goorani et al., 2020). In our study, FTIR spectrum was used to detect potential biomolecules present in Citrus lemon aqueous leaf extract responsible for reducing Ag-NPs. Fig. 1 shows the FT-IR spectrum of Ag nanoparticles containing *Citrus lemon* leaf aqueous extract in the wavenumber of 400–4000 cm^{-1}. In Fig. 1, wavenumber 3408 is due to hydroxyl and phenolic groups. The bands at 2915, 1367–1618, 1084, and 497 cm^{-1}

are referred to as asymmetric CH_2 stretching, aromatic rings, C-O stretching and Ag-O, respectively.

FE-SEM analysis is a method for assessing the size and morphology of materials such as Ag nanoparticles and is extensively utilized in chemistry, physics, and biology investigations (Zangeneh, 2020; Mohammadi et al., 2020; Hamelian et al., 2020) In this study, the FE-SEM image of Ag nanoparticles containing *Citrus lemon* seed aqueous extract is shown in Fig. 2. In this figure, Ag nanoparticles are spherical, with an average size of 25.1 nm. It seems that few Ag-NPs form an agglomerated structure because of the presence of hydroxyl groups.

Transmission Electron Microscopy (TEM) is a technique using an electron beam to provide images of an NP sample with higher resolution in comparison with light-based imaging techniques. As the preferred technique, TEM is employed to measure NP size, grain size, size distribution and morphology directly (Zangeneh, 2020; Mohammadi et al., 2020; Shahriari et al., 2019; Goorani et al., 2020). Fig. 3 shows that Ag nanoparticles containing *Citrus lemon* seed extract are spherical and, on the average, 25.1 nm in size. Furthermore, a small number of them can be seen in other forms.

Fig. 4 reveals the UV–Vis spectra analysis for *Ag nanoparticles* containing *Citrus lemon* seed aqueous extract. In this study, UV–Vis spectroscopic analysis indicated an absorption peak at 417 nm. Formation of Ag-NPs at this peak is supported by a lot of research indicating 400–450 nm as the range of wavelengths for Ag-NP formation from medicinal plants (Zangeneh, 2020; Mohammadi et al., 2020; Hamelian et al., 2020; Hemmati et al., 2019).

3.2. Antioxidant and cytotoxicity of Ag nanoparticles containing Citrus lemon aqueous extract

Using DPPH test, we assessed antioxidant properties of silver nanoparticles (Ag-NPs) green-synthesized by Citrus lemon aqueous seed extract.

In this study, we assessed antioxidant properties Ag-NPs green-synthesized by Citrus lemon leaf aqueous extract by suing DPPH Test, which is used to evaluate free radical scavenging activities of different antioxidant materials. This approach depends on the reduction of free radical DPPH by antioxidants in the absence of other free radicals and this action generates a color based on which absorption intensity can be evaluated by spectroscopy. The highest light absorption of DPPH radical was observed at 515–520 nm, at which methanolic solution is purple and acts as an electron absorber of a donor molecule like an antioxidant, thus changing DPPH to DPPH2. Consequently, purple color of the solution becomes yellow; therefore, absorption intensity decreases at 515–520 nm. We can find out the importance of antioxidant activities by evaluating the absorption intensity reduction through spectroscopy (Zangeneh, 2020; Mohammadi et al., 2020; Hamelian et al., 2020; Hemmati et al., 2019; Jalalvand et al., 2019; Shahriari et al., 2019; Goorani et al., 2020).

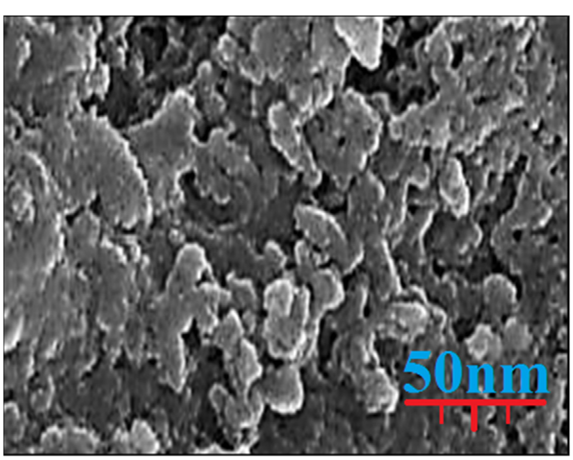

Fig. 2 FE-SEM image of silver nanoparticles green-synthesized by *Citrus lemon* leaf.

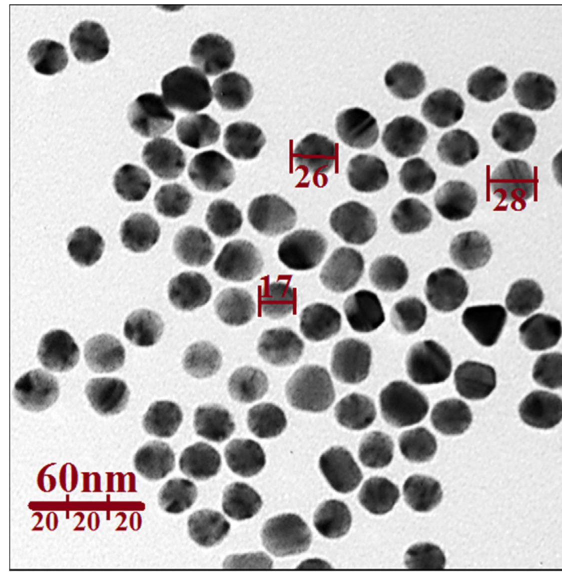

Fig. 3 TEM image of silver nanoparticles green-synthesized by *Citrus lemon* leaf.

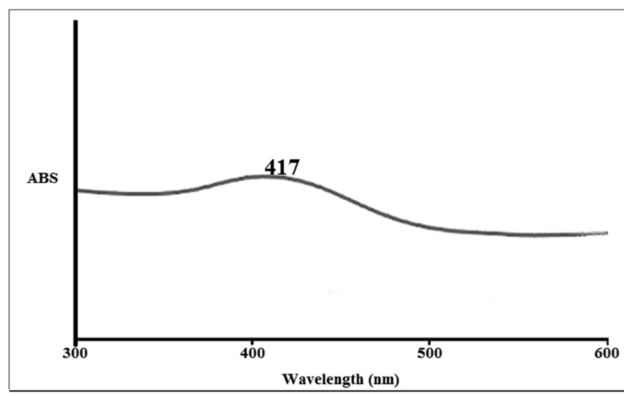

Fig. 4 UV–Vis spectrum of silver nanoparticles green-synthesized by *Citrus lemon* leaf.

Fig. 5 illustrates scavenging capacity of BHT and Ag-NPs green-synthesized by Citrus lemon aqueous leaf extract at different concentrations expressed as percent inhibition.

In the antioxidant test, IC50 of *Citrus lemon* leaf aqueous extract, Ag nanoparticles and BHT were 373, 189, and 215 μg/mL, respectively (Table 1).

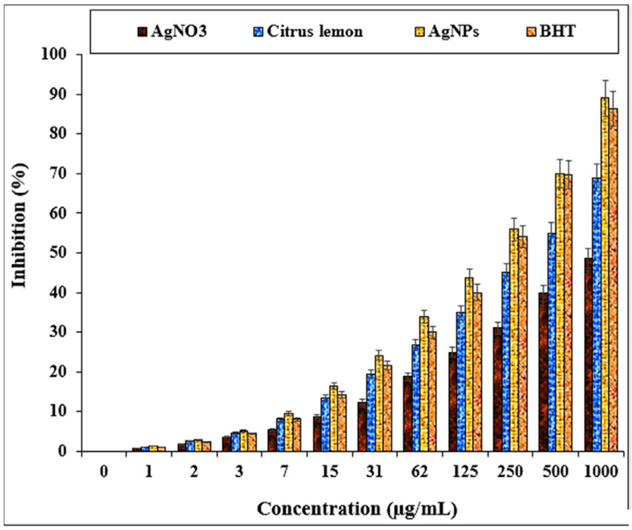

Fig. 5 Antioxidant properties of $AgNO_3$, *Citrus lemon* leaf aqueous extract, Ag nanoparticles and BHT against DPPH.

Studies have shown that antioxidant features of metallic nanoparticles green-synthesized by medicinal herbs are more significant than other metal nanoparticles. Considerable antioxidant activities of metallic nanoparticles green- synthesized by several medicinal herbs like *Gundelia tournefortii* L., *Allium noeanum* Reut. Ex Regel, *Falcaria vulgaris*, *Thymus vulgaris* and *Camellia sinensis* have been already confirmed. Ag nanoparticles green-synthesized by medicinal herbs show noticeable antioxidant activities against the formation of free radicals in the living system. Green-formulated Ag-NPs have important redox activities and play a noticeable role in scavenging free radicals (Zhaleh et al.,2019; Shahriari et al., 2019; Goorani et al., 2020).

Research has shown that phenolic and flavonoid compounds added to metallic nanoparticles had important antioxidant activities (Zangeneh, 2020; Shahriari et al., 2019; Goorani et al., 2020).

As mentioned in the introduction, *Citrus lemon* contains antioxidant compounds. Antioxidant molecules of *Citrus lemon* are α-Pinene, Sabinene, β-Pinene, Δ-3-Carene, α-Terpinene, p-Cymene, Limonene, *cis*-Ocimene, γ-Terpinene, Linalool oxide, α-Terpinolene, Linalool, *cis*-p-Menth-2-en-1-ol, Terpinen-4-ol, α-Terpineol, Linalyl acetate, Geraniol, α-Terpinyl acetate, Piperitenone oxide, Neryl acetate, Geranyl acetate, β-Elemene, trans- Caryophyllene, β-Farnesene, α-Humulene, Germacrene D and α-Farnesene (Al-Jabri et al., 2014; Di Vaio et al., 2010; Espina et al., 2011; Golmakani and Moayyedi, 2015).

MTT is a calorimetric assay used to examine metabolic activity of cells (Arulmozhi et al., 2013). NAD (P) H-dependent cellular oxidoreductase enzymes reflect the number of viable cells present under specified conditions. Such enzymes are capable of reducing tetrazolium dye MTT 3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide to its insoluble formazan with purple color. Other dyes, including XTT, MTS and WSTs, closely related to teltrazolium are used with 1-methoxyohenazine meth sulfate (PMS) as the intermediate electron acceptor. With cell-impermeable WST-1, reduction takes place outside cells via plasma membrane electron transmission [15–16, 18]. Nevertheless, this traditionally accepted explanation is contended currently due to newly found evidence of MTT reduction to formazan with lipid cellular structures with no obvious involvement of oxidoreductases (Arulmozhi et al., 2013).

Measurements of cytotoxicity (loss of viable cells) or of cytostatic activity (shift from proliferation to quiescence) of potential medicinal agents and toxic materials can be performed by tetrazolium dye assays. Since MTT reagents are sensitive to the light, respective assays are usually performed in the dark (Arulmozhi et al., 2013).

In this study, the cells treated with different concentrations of the present silver salt, *Citrus lemon* leaf aqueous extract and Ag nanoparticles were assessed by MTT assay for 48 h in terms of the cytotoxicity effects on normal (HUVEC) cell lines. Absorbance rates were evaluated at 570 nm, which represented viability of a normal cell line (HUVEC) even up to 1000 µg/mL for silver salt, *Citrus lemon* leaf aqueous extract and Ag nanoparticles (Fig. 6).

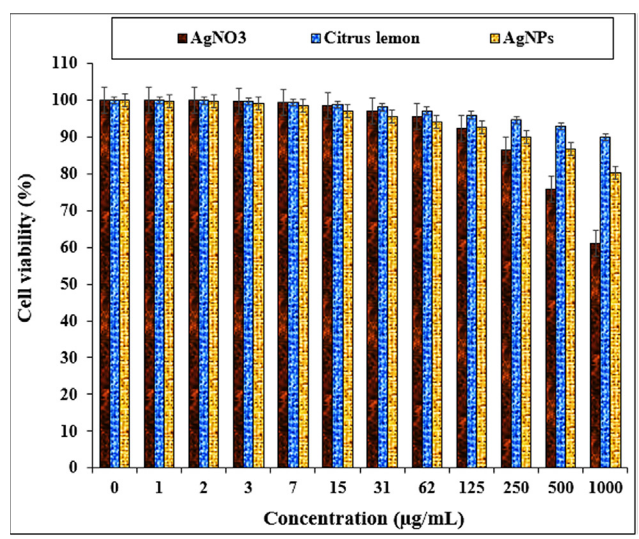

Fig. 6 Cytotoxicity properties of $AgNO_3$, *Citrus lemon* leaf aqueous extract and Ag nanoparticles against HUVEC cell line.

Table 1 IC50 of $AgNO_3$, *Citrus lemon* leaf aqueous extract, Ag nanoparticles and BHT obtained by antioxidant test.

	$AgNO_3$ (µg/mL)	*Citrus lemon* (µg/mL)	Ag nanoparticles(µg/mL)	BHT (µg/mL)
IC50 against DPPH	–	373	189	215

3.3. Cutaneous wound healing potentials of Ag nanoparticles containing Citrus lemon aqueous extract

Physical injuries create wounds leading to opening or breaking of skin including punctured, scratched, scraped and cut skin. As a result of failed healing phases, wounds are likely to become chronic (Mussel et al., 2003). Although human body is generally equipped with a self-healing defense mechanism, wound healing process can be delayed under such critical conditions as microbial infections. In some cases, a lot of bodily pains and costly treatments result from wound complexity (Kream and Stefano, 2010; Bolboacă and Jäntschi, 2008; Mandal et al., 2007; Abolhassani, 2010).

Wound dressing is considerably effective in the healing process. Duration of healing time is shortened and chances of scar formation are reduced only if wounds are covered properly. The main purpose of dressing wounds is to accelerate wound healing process by preventing microbes from growing, absorbing wound fluid and keeping a wet environment around wounds (Guo and DiPietro, 2010; Souba and Wilmore, 1999; Hajialyani et al., 2018). Some antimicrobial activity is exhibited by plant extracts potentially, reducing the rate of growth of microorganisms. Medicinal herbs covering the skin are absorbed by body, curing a wide range of diseases such as arthritis, skin wounds and infections as well as diseases like hypertension, asthma and diabetes if the herbs are used orally (Guo and DiPietro, 2010; Souba and Wilmore, 1999). Some examples of medicinal plants used for treating wounds are *Nyctanthes arbor-tristis* L., *Momordica charantia* L., *Bryonia laciniosa* L., *Biophytum sensitivum*, *Coleus forskohlii*, *Piper nigrum*, *Aloe vera*,*Chromola enaodorata*, *Indigofera aspalathoides* DC., *Fraxinus angustifolia*, *Ficus religiosa*, *Curcuma longa* L., *Cassia roxburghii*, *Trigonella foenum-graecum*, *Aza-*

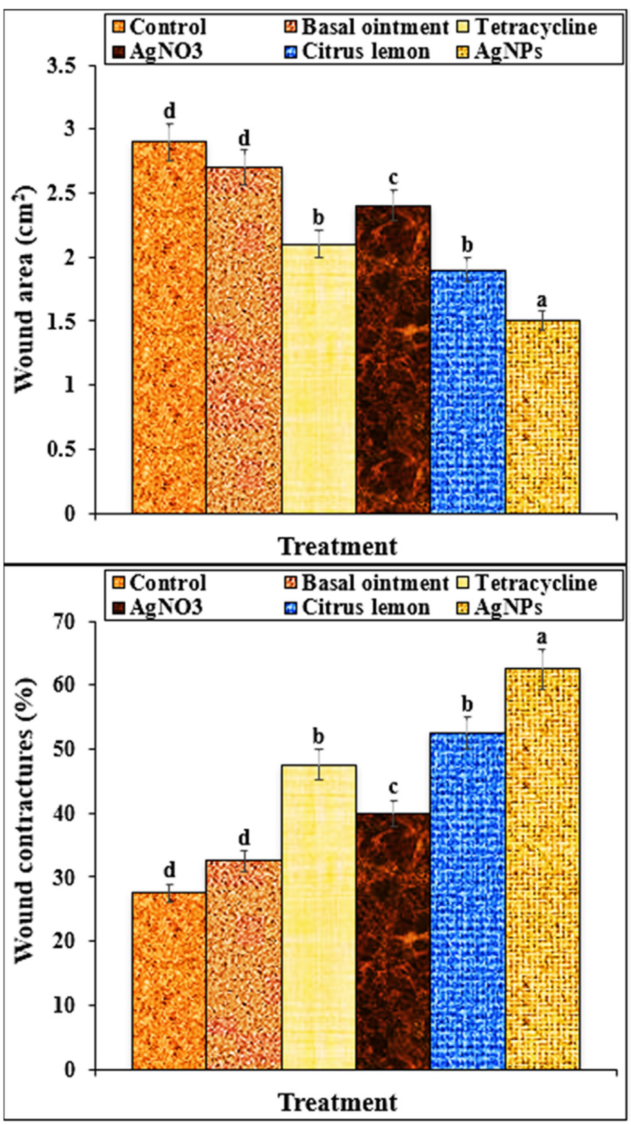

Fig. 7 The levels of wound area and wound contracture in groups treated with by basal, tetracycline, $AgNO_3$, *Citrus lemon* leaf aqueous extract and Ag nanoparticles ointments. Different letters show a significant difference between test groups ($p \leq 0.01$).

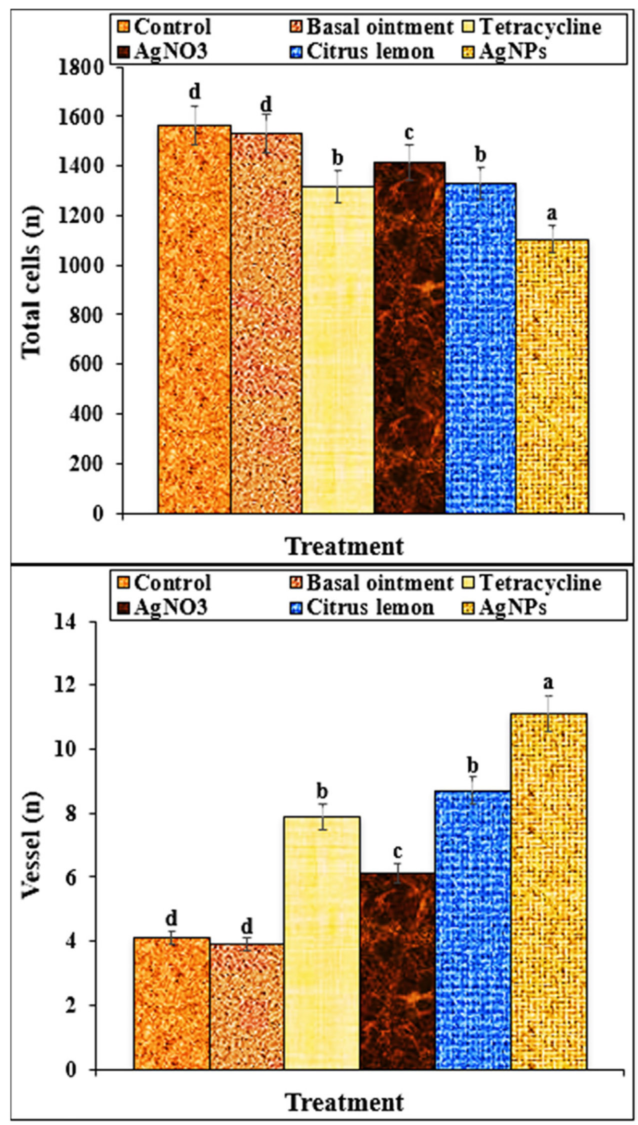

Fig. 8 The number of total cells and vessels in groups treated with basal, tetracycline, $AgNO_3$, *Citrus lemon* leaf aqueous extract and Ag nanoparticles ointments. Different letters show a significant difference between test groups ($p \leq 0.01$).

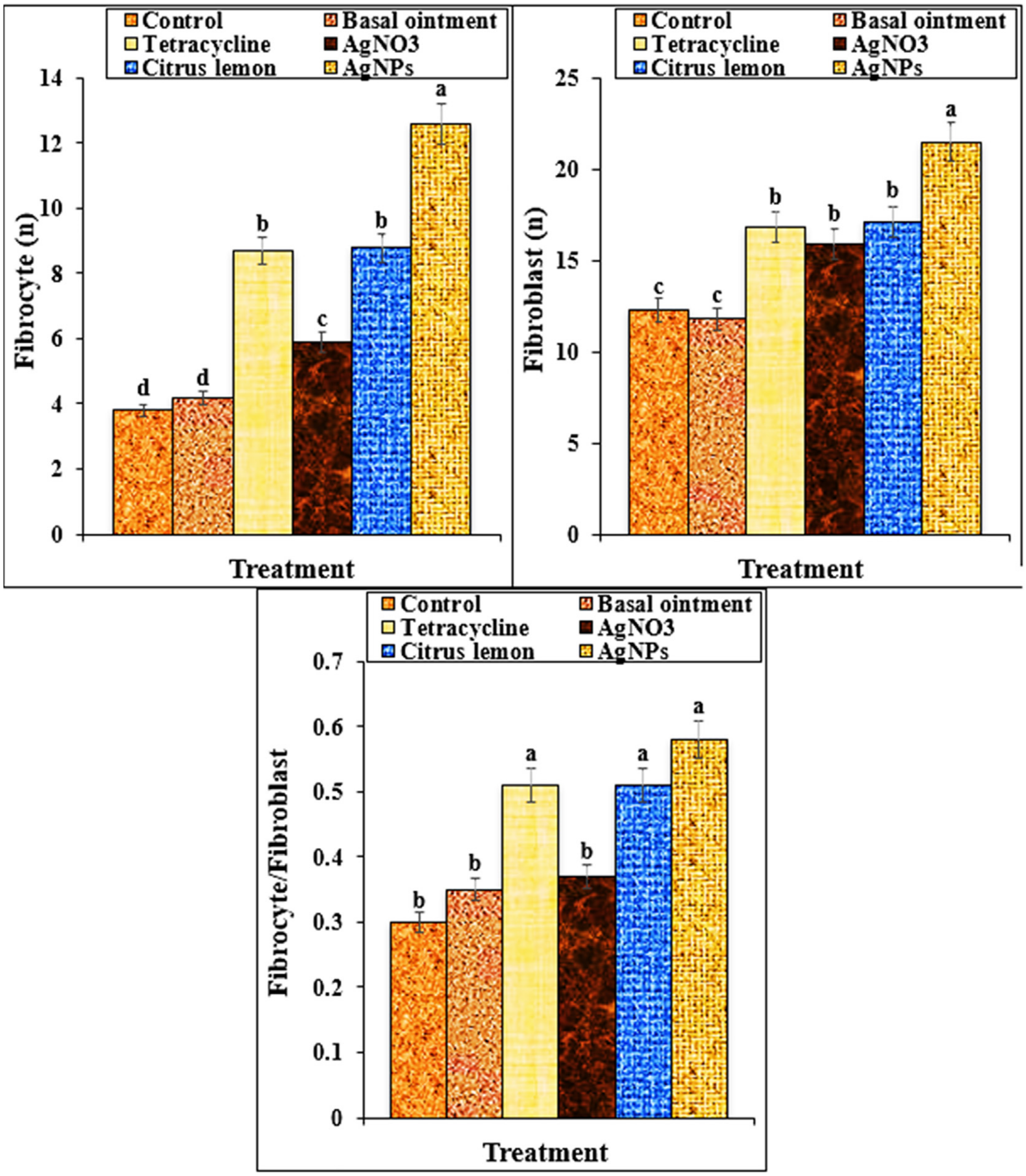

Fig. 9 The number of fibrocytes and fibroblasts and the ratios of fibrocyte/fibroblast in groups treated with basal, tetracycline, $AgNO_3$, *Citrus lemon* leaf aqueous extract and Ag nanoparticles ointments. Different letters show a significant difference between test groups ($p \leq 0.01$).

dirachta indica A., *Lansium domesticum* Corrêa, *Citrus reticulate* and *Centella asiatica* (Hajialyani et al., 2018).

Results obtained from macroscopic, microscopic and biochemical studies showed that Ag nanoparticles ointment significantly decreasesd/ increasesd the percent of wound area, the numbers of total cells, neutrophils and lymphocytes/ the percent of wound contracture; numbers of blood vessels, fibrocytes and fibroblasts, fibroblast/ fibrocyte ratios, and concentrations of hydroxyproline, hexosamine and hexuronic acid compared to other study groups (Figs. 7–11).

As shown by studies, the amount of angiogenesis at the wound site increased as the wound appeared, causing more fibroblasts to migrate to the site.

Then, fibroblasts induced production of collagen (proline, hydroxyl proline, glycine) and of extracellular matrix compounds like hexosomine and hexuronic acid (Azhdari-Zarmehri et al., 2014). After that, fibroblasts changed into fibroecytes. Effectiveness of fibrocytes in healing wounds was far higher than that of fibroblasts (Azhdari-Zarmehri et al., 2014; Caetano et al., 2016).

Moreover, inflammatory cells, i.e., macrophages, neutrophils and lymphocytes, migrated to the wound site after angiogenesis (Dwivedi et al., 2017; Nayak et al., 2007). High activities of inflammatory cells increased the presence of pus in the wound site. Antioxidant compounds such as metallic nanoparticles reduced extra activities and accumulation of

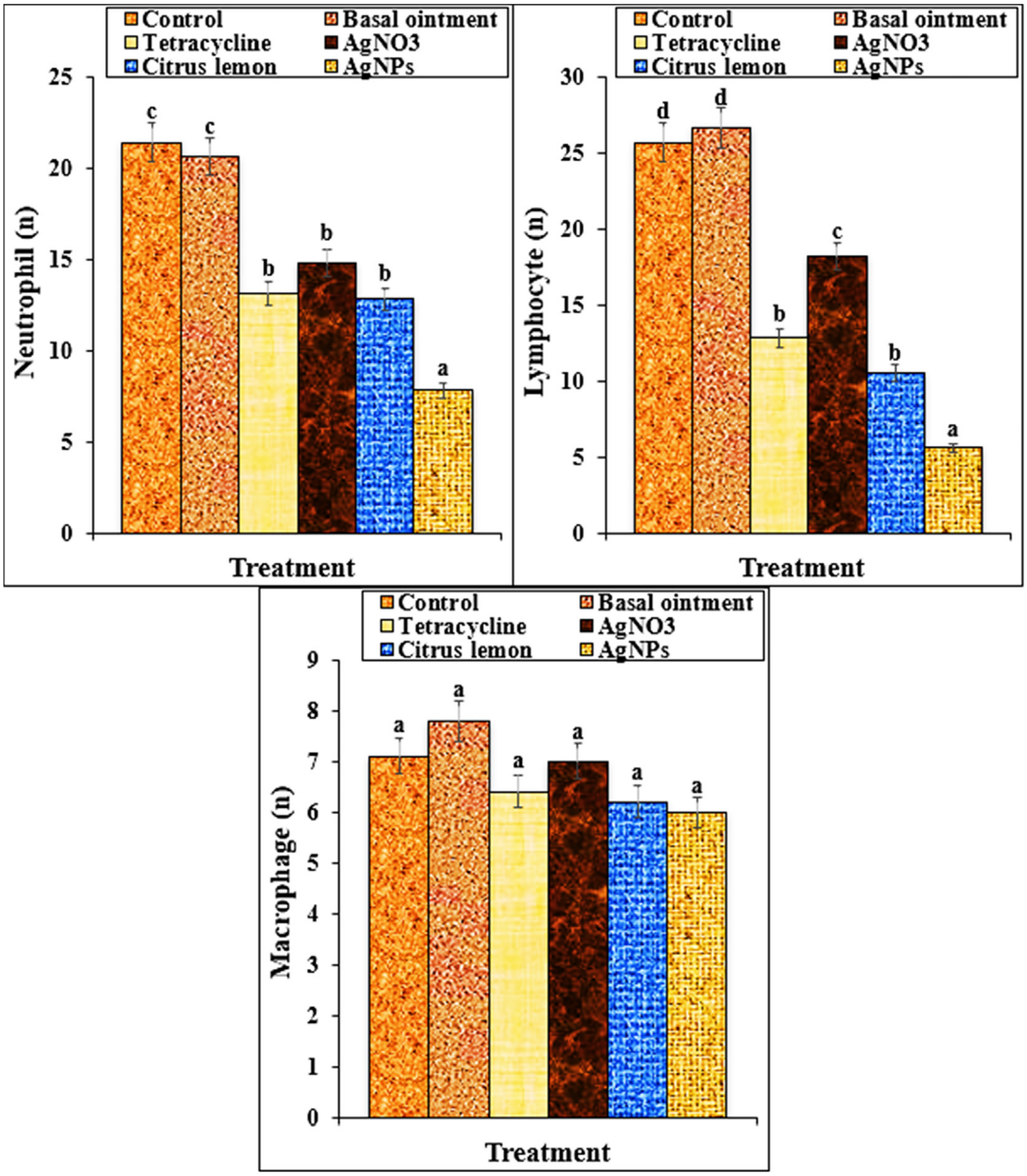

Fig. 10 The numbers of neutrophils, lymphocytes and macrophages in groups treated with basal, tetracycline, $AgNO_3$, *Citrus lemon* leaf aqueous extract and Ag nanoparticles ointments. Different letters show a significant difference between test groups ($p \leq 0.01$).

metallic nanoparticles in the wound site (Koh and DiPietro, 2011).

4. Conclusions

In present research, Ag nanoparticles were generated by the reaction between $AgNO_3$ and *Citrus lemon* leaf aqueous extract under in-vitro conditions. TEM, FE-SEM, UV–Vis and FT-IR methods were utilized to characterize nanoparticles. Base on FT-IR spectra, the presence of a great number of the antioxidant compounds provided appropriate conditions for the reduction of silver. It was found by TEM that 25.1 nm was a favorable mean size of Ag-NPs. Ag nanoparticles showed the best antioxidant activities against DPPH. But they did not have any cytotoxicity effects on the human normal cell line (HUVEC). In the cutaneous wound healing experiments, application of Ag-NP ointment improved parameters of cutaneous wound healing significantly ($p \leq 0.01$), so that they resulted in a decrease in the wound area, the numbers of total cells, neutrophils and lymphocytes and in an increase in the wound contracture, in the numbers of vessels, fibrocytes fibroblasts and their ratios, and in the amounts of hexosamine, hydroxyl proline and hexuronic acid significantly ($p \leq 0.01$) compared to other male rat groups. Based on the results from previously performed studies, which showed that silver nanoparticles (Ag-NPs) had a wide variety of therapeutic and antibacterial properties effective in controlling wound infection and healing wounds, and, also, on the results from present study, in case Ag-NPs containing Citrus lemon leaves are studied clinically thoroughly, they may be applied as a highly efficient drug to treat cutaneous wounds in humans.

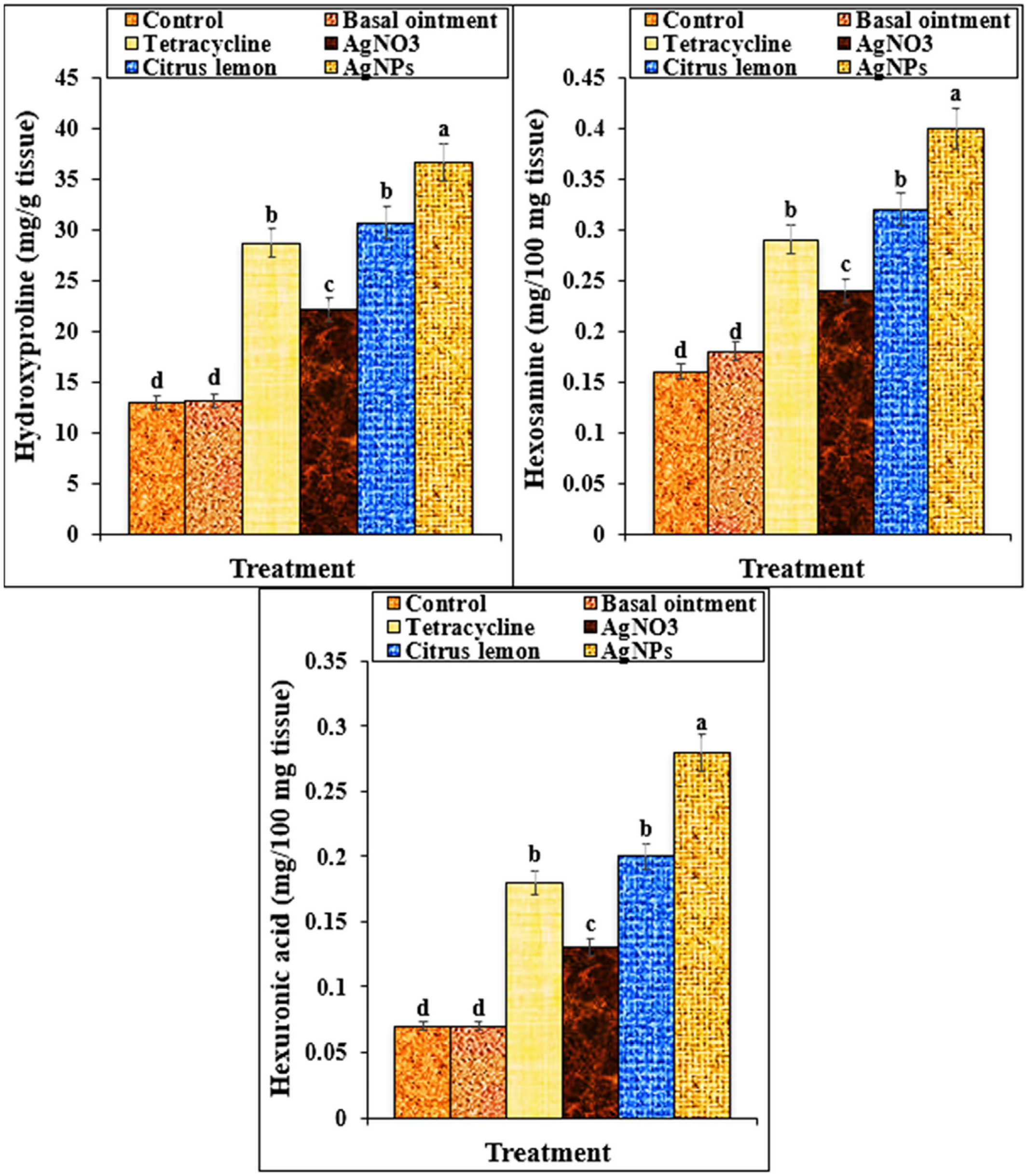

Fig. 11 Concentrations of hydroxyproline, hexosamine and hexuronic acid in groups treated with basal, tetracycline, $AgNO_3$, *Citrus lemon* leaf aqueous extract and Ag nanoparticles ointments. Different letters show a significant difference between test groups ($p \leq 0.01$).

5. Limitation

There was no limitation in this study.

Before starting to work, the project partners tried to coordinate as much as possible to provide the necessary facilities and materials, so that we did not face any particular problems during the work, but in some cases, our tasks were somewhat time-consuming, for example, transfer of nanoparticles to other provinces to be analyzed, histological examinations, and the time passed before receiving the results. In general, there were no specific problems or restrictions during the project.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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