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Development of PLGA wound dressing containing silver nanoparticles synthesized from licorice extract

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Abstract

Nanofiber wound dressings are developed using various polymers and adding different active agents to the polymers during the production phase. In this study, PLGA, PLGA containing silver nanoparticles (AgNP/PLGA), PLGA nanofibers containing 1%, 2%, 3% licorice root, and silver nanoparticle doped (LR-AgNP/PLGA) were produced by electrospinning method. The green synthesis method was used to produce licorice-containing silver nanoparticles (LR-AgNP). The morphological analysis of nanofibers was determined using diameter measurements on SEM images. The average fiber diameters of PLGA, AgNP/PLGA, and LR-AgNP/PLGA nanofibers are 344 nm, 260 nm, and 357 nm, respectively. FTIR analysis was used for the determination of chemical bonds. The FTIR graph of PLGA, AgNP/PLGA and LR-AgNP/PLGA nanofibers showed similar peaks in the 3008-2883 cm^{-1}, 1500-1412 cm^{-1}, 1181-1086 cm^{-1} and 1755-1750 cm^{-1} bands. The wound healing potential of the produced nanofibers was evaluated on an *in vitro* wound scratch model. According to the results obtained, LR-AgNP/PLGA nanofibers showed the fastest wound closure. As a result of these studies, it was determined that PLGA nanofibers with silver nanoparticles containing licorice root extract could accelerate the wound healing process.

Introduction

The wound, deterioration of skin integrity as a result of tissue destruction caused by mechanical, biological, physical or chemical traumas. In addition, there may be many acute or chronic causes of wounds that may occur due to chronic diseases such as diabetes, peripheral vascular diseases that reduce the quality of life of the patient (Dong & Guo, 2021). Although the process of wound healing is highly complex and depends on the delicate interaction of numerous factors, wound healing in normal processes generally consists of four successive stages. These stages include hemostasis, inflammation, proliferation and finally wound remodeling (Reinke & Sorg, 2012).

There are many methods used in wound treatments. In addition to conventional ointments, creams, gels or traditional wound bandages, modern wound dressings and drug delivery systems are also used in wound treatment. Apart from these, newly developing therapies such as stem cell therapy, gene therapy, photothermal and photodynamic therapy have also started to be used in recent years (Liang et al., 2021). Types of wound dressings include synthetic, semi-synthetic and natural wound dressings. An ideal wound dressing has features such as oxygen and water vapor permeability, ability to absorb wound exudate, ability to adhere to the wound surface, antibacterial, elasticity and easy application, etc. (Zeng et al., 2022). There are many types of wound dressings produced for this purpose. According to the material they are produced from, the main modern wound covers include

hydrocolloids, alginate covers, hydrogels and nanofiber wound covers. Nanofiber wound covers can be produced using various methods. Electrospun nanofibers have high porosity, high surface area-to-volume ratio, and ability to perfectly mimic extracellular matrix (ECM) (Zhou et al., 2022). There are many advantages of electrospinning such as the production of very fine fibers with a large surface area on the order of a few nanometers is the ease with which they can be functionalized for various purposes, superior mechanical properties and ease of operation. Electrospun wound dressers can be modified by using healing agents together with various polymers (Liu et al., 2022). PLGA (Poly (lactic-co-glycolic acid)), one of the synthetic polymers used in electrospinning, is approved for clinical applications by the U.S. Food and Drug Administration (FDA) (Muddineti & Omri, 2022). PLGA is a promising material for tissue engineering due to its good mechanical properties, non-toxic biodegradation products and adjustable biodegradation time (Zhao et al., 2016).

Silver has been used as a healing agent by civilizations around the world for thousands of years. Silver is the most studied precious metal for use in combating bacterial infections (Dowsett, 2004). Studies have shown that silver products with sustained silver release have a bactericidal effect on the wound that allows effective management of odor and exudate, thus reducing the risk of colonization and preventing infection (Nephew, 2003). The nano-sized form of silver used in nanofiber structures are silver nanoparticles (AgNP). AgNPs are used in wound care to prevent secondary infections as they are effective against a spectrum of microbes that can slow down the healing process (Pal et al., 2017). There are many methods used for the production of AgNPs. The production method that has developed in recent years is AgNP production with green synthesis. The green synthesis method of AgNPs has the advantages of being non-toxic, safe for humans, environmentally friendly and economically viable compared with chemical and physical synthesis methods (Mohanpuria et al., 2008).

Medicinal and aromatic plants are used in many fields such as food, health, cosmetics, paint, textile, medicine, agriculture. Natural raw plant extracts are used in the treatment of many diseases in Turkey. Herbal products are also used in wound treatment depend on anti-inflammatory, antioxidant, antimicrobial features (Shedoeva et al., 2019). Licorice root is used in many areas of medicine and supports many biological activities such as cell regeneration due to its support of growth factors thanks to its bioactive components (Damle, 2014).

Keratinocytes are one of the cells found densely in the structure of the skin, the outermost layer of the body. Keratinocytes constitute 90% of the cells in the epidermis. They are of great importance in the wound healing stages due to their regular production of many antimicrobial peptides known for their innate immune defense functions, usually in response to injury or infection (Chan & Shi, 2022). HaCaT cell culture is the process of growing cells obtained from human skin in an artificial environment. Cells can be cultured directly from the tissue or by dissecting them using enzymatic and mechanical methods. After the cultured cells show sufficient proliferation, they can be used in *in vitro* studies (Stacey, 2001).

In this study, the preparation of licorice extract, antioxidant and antibacterial properties including characterization tests, are carried out and *in vitro* cell culture method is aimed to determine the wound healing property. For this, firstly licorice root extract was prepared, and the antioxidant capacity of this extract was determined. The fibrous form of the roots of the licorice plant was used to prepare the licorice root extract. Then, AgNPs (LR-AgNP) containing licorice root were obtained by green synthesis method using licorice extract. These LR-AgNPs are incorporated into the structure of PLGA during the electrospinning phase. In the continuation of the study, pure PLGA, PLGA (AgNP/PLGA) containing commercial AgNP, PLGA (LR-AgNP/PLGA) nanofibers containing AgNP synthesized with licorice root at the rates of 1%, 2% and 3% were produced by electrospinning method for use as wound dressing. Characterization tests of the nanofiber wound dressings were performed and their applicability on an *in vitro* wound scratch model was evaluated. Scanning Electron Microscopy (SEM) images, Fourier-Transformed Infrared Spectroscopy (FTIR), X-Ray Diffraction (XRD), antibacterial and antioxidant activity analyses were done for characterization of material. Nanofibers, which were determined to be antibacterial, were tested on *in vitro* scratch models and the wound closure rates were evaluated by imaging at every 3-hour intervals.

Materials and Methods

PLGA polymer was obtained from Shenzhen Esun Industrial Co. in China. $AgNO_3$, N, N-Dimethylformamide (DMF), Dichloromethane (DCM) used in the experiments were obtained from Carlo Erba. Commercially available pure AgNP was obtained from BRK Laboratory Materials Chemical Products company.

Preparation of licorice extract and green synthesis of LR-AgNPs

Licorice extract was prepared by mixing the powdered licorice root and pure water in a magnetic stirrer for 24 h. The amount of $AgNO_3$ and extract to be used for the production of licorice silver nanoparticles (LR-AgNP) to be produced by green synthesis was determined by quantification with UV spectrophotometer and the ratio of 7:3 (extract/$AgNO_3$) was decided for production. The specified ratio of $AgNO_3$ and licorice extract was mixed in the dark for 24 h. The mixture was centrifuged and the part that settled

to the bottom was dried in the oven and LR-AgNPs were obtained in powder form (Shameli et al., 2012).

Nanofiber production by electrospinning

Solutions of nanofibers to be produced by electrospinning method was prepared. For pure PLGA nanofiber solution, PLGA's solvents DCM/DMF were mixed at a ratio of 50:50 v/v. 20% w/v PLGA polymer added to this mixture. The PLGA polymer was mixed with a magnetic stirrer until it was completely dissolved. For the AgNP/PLGA solution, the solution was prepared in the same way, 1% of the PLGA ratio AgNP was added and mixed with a magnetic stirrer until it became homogeneous. In the same way, 1%, 2%, and 3% LR-AgNP/PLGA solutions were prepared and all of them were drawn into individual syringes and placed in the electrospinning device. A large number of nanofibers wound dressings have been produced by adjusting different parameters (Öktemer & Avcı, 2021).

Characterization tests

SEM image analysis, FTIR, XRD, and antibacterial analysis were performed for the produced nanofibers. In addition, the antioxidant activity of the licorice root extract used in the study was determined. SEM image analysis was performed by Ege MATAL. In order to examine the fiber diameter thickness and fiber distribution of nanofibers, nanofibers were cut into 1 cm^2 circles and their images were taken with the help of a SEM.

The crystal structures of the produced nanofibers and LR-AgNP were analyzed by XRD analysis. XRD measurements were recorded at 1% min scanning speed and 0.05° step size. XRD analyses were performed by Ege University Drug Development and Pharmacokinetics Research-Application Center (Arge-far).

FTIR analysis was performed to analyze the functional groups in the chemical structure of the produced nanofibers. FTIR analysis is based on the analysis of the absorption spectrum of atomic and molecular bonds of nanofibers exposed to infrared rays. This analysis was also performed by Ege MATAL.

Disk diffusion method was used for antibacterial activity analysis. This analysis was performed for three different nanofiber samples (PLGA, AgNP/PLGA, LR-AgNP/PLGA). The analysis was performed by Ege MATAL. In the study, *Escherichia coli* (*E. coli*) ATCC 25922 was used as gram-negative bacteria and *Staphylococcus aureus* (*S. aureus*) ATCC 25923 was used as gram-positive bacteria. The samples were cut into circular shapes with a diameter of 10 mm and sterilized bidirectionally under UV light for 1 h. Then, the samples were placed in petri dishes and waited for 15 min and incubated at 37 ^{o}C for 16-24 h. After incubation, the zones formed by the sample were measured. The antibacterial properties of the samples and the inhibition zone diameters formed are directly proportional.

The antioxidant activity of the licorice root extract used in the study was determined by the Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method. The antioxidant activity of licorice root was evaluated according to two different parameters. The first of these is ascorbic acid equivalent. Since ascorbic acid has high antioxidant properties, a high ascorbic acid equivalent shows that the material is antioxidant. The second parameter used to determine antioxidant activity is DPPH % removal. DPPH% removal is the parameter used to determine the antioxidant properties of herbal extracts, pure antioxidant molecules and phenolic compounds. A high DPPH % removal value shows that antioxidant activity is high (Seyhan, 2019).

***In vitro* cell culture studies**

The wound dressings produced were tested with *in vitro* scratch test. Human keratinocyte (HaCaT) cells were used in cell culture studies. In order to create the scratch model, cells were seeded in 24-well plates with 1×10^5 cells per well, provided that they showed at least 80% viability (Ker-Woon et al., 2015). As stated in the literature, when keratinocyte cells filled all the wells, a scratch was made in the middle of each well to create a scratch wound model (Martinotti & Ranzato, 2019). Wound closure rates were evaluated by imaging every 3 h for 48 h (Ker-Woon et al. 2015). Experiments were carried out with six groups and three repetitions. The groups were as follows; control, PLGA, AgNP/PLGA, 1%, 2%, and 3% LR-AgNP/PLGA. The healing percentages of the wound models whose photographs were taken due to wound closure were calculated using the Image J program. One-way ANOVA test was performed for statistical comparisons in wound closure groups; Tukey analysis was used for comparisons between groups.

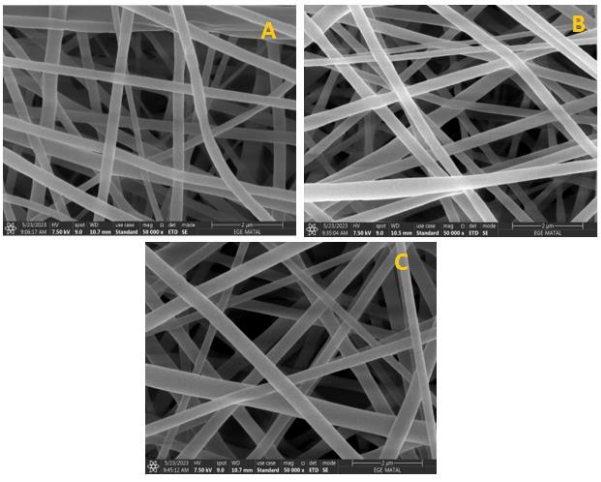

Figure 1. SEM images of nanofibers **A)** PLGA nanofiber, **B)** AgNP/PLGA nanofiber, **C)** LR-AgNP/PLGA nanofiber.

Results and Discussion

SEM images of nanofibers are shown in Figure 1. The addition of AgNP to the pure PLGA resulted in a decrease in the fiber diameter of the nanofiber. The

average diameters of LR-AgNP/PLGA fibers were measured as 357 nm. Fiber diameter distribution is in the range of 210-520 nm (Table 1). In the literature, they examined the fiber diameter change in the case of adding AgNP to different PLGA varieties and determined that the fiber diameter of PLGA nanofiber, which was calculated as 781 ±130 nm, decreased to 538 ± 72 nm when AgNP was added. It has been stated that this decrease is due to the addition of AgNPs with high conductivity to the material (Gora et al., 2015). In another study, it was determined that when AgNP was added to cellulose acetate fibers, the average nanofiber diameter decreased from 208 nm to 136 nm (Kumar et al., 2023). In our study, the fiber diameters of AgNP/PLGAs are distributed in the range of 110-350 nm (Table 1). The average fiber diameter was calculated as 260 nm. It has been determined that there is a decrease in the average fiber diameter relative to PLGA nanofibers.

Table 1. Fiber diameter distribution of nanofibers

Nanofiber Type	Average Fiber Diameter	Fiber Diameter Distribution
PLGA	344 nm	200-600 nm
AgNP/PLGA	260 nm	110-350 nm
LR-AgNP/PLGA	357 nm	210-520 nm

FTIR was measured to determine functional groups in the resulting nanofibers. The result of FTIR analysis is due to the different chemical bonds in each peak nanofiber structure formed in the specified graph (Figure 2). The FTIR graph of PLGA, AgNP/PLGA, and LR-AgNP/PLGA nanofibers showed similar peaks in the 3008-2883 cm^{-1}, 1500-1412 cm^{-1}, 1181-1086 cm^{-1}, and 1755-1750 cm^{-1} bands. There are no significant differences in the FTIR spectral analysis of nanofibers, and they have similar peak points. Some shift in the peaks cm^{-1} occurred with the addition of AgNPs and LR-AgNPs to the PLGA nanofiber structure. In the FTIR analysis of a study conducted with PLGA nanofibers, they determined significant peaks and when these spectrum peaks were examined, they stated that bands could be formed at 1754 cm^{-1} from the C=O stretch, at 1185 cm^{-1} from the stretching of the C-O-C bond, and at 1130 cm^{-1} from the C-O bond and 1453 cm^{-1} from the C-H bond, respectively (Khan et al., 2018). When the FTIR analysis of the PLGA nanofiber in our study was examined, it can be said that the bands at 1183 cm^{-1} were caused by the stretching of the C-O-C ether group, and the strong characteristic absorption bands at about 1755 cm^{-1} were caused by the stress vibration of the C-O bond. It can be said that the peaks at 1452 cm^{-1} and 1130 cm^{-1} are due to the C-H bond and the C-O bond, which are the methyl group of PLGA, respectively. In the study of Schoeller et al. (2021), it was determined that while there was a change in the percentage of permeability in the PLGA polymer added to AgNP, there was no change in the wavenumbers in FTIR spectroscopy. In our study, the addition of AgNPs to the PLGA polymer did not reveal a very significant difference in FTIR spectroscopy. The shifts in the peaks of LR-AgNP/PLGA nanofiber compared to PLGA and AgNP/PLGA nanofibers in FTIR analysis may be due to the physical interactions of licorice extract with nanofibers.

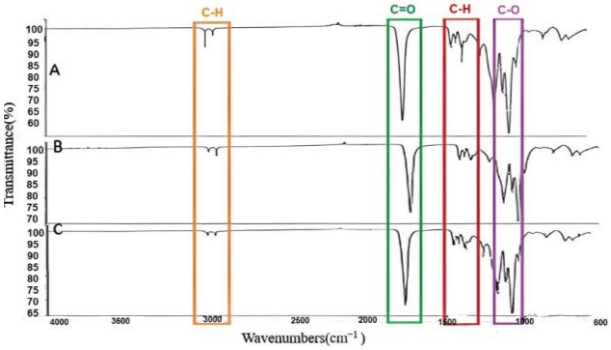

Figure 2. FTIR graphs of nanofiber structures **A)** PLGA nanofiber, **B)** AgNP/PLGA nanofiber, **C)** LR-AgNP/PLGA nanofiber.

Examined crystal structures with XRD analysis of nanofibers. In XRD analysis, PLGA, AgNP/PLGA, LR-AgNP/PLGA, and LR-AgNP showed the highest peak values at 2θ=20.92°, 43.98°, 16.68°, and 38.12°, respectively. When compared in terms of peak density, PLGA in nanofiber structure, LR-AgNP/PLGA have a lower peak density, while LR-AgNPs in nanoparticle structure have a higher peak density (Figure 3, Figure 4). In addition, the peak at 43.98° in the graph of AgNP/PLGA represents one of the reflection planes of Ag's Bragg reflections (111), (200), (220), and (311). This shows that AgNPs are dispersed into the nanofiber (Chen et al., 2023).

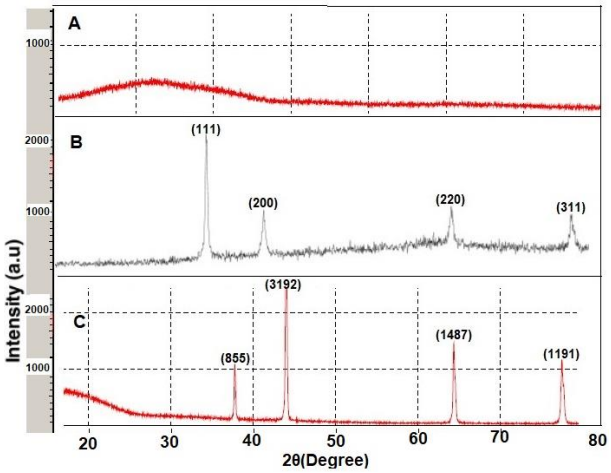

Figure 3. XRD plots **A)** PLGA, **B)** AgNP (Chook et al., 2012), **C)** AgNP/PLGA.

The antibacterial properties of the PLGA, AgNP/PLGA, and LR-AgNP/PLGA nanofibers we obtained were investigated against *E. coli* and *S. aureus* bacteria by applying the disk diffusion test at 37 °C for 24 h of incubation. The nanofibers formed zones around the sample by showing different antibacterial effects against *E. coli* and *S. aureus* bacteria. The antibacterial effects were evaluated by measuring the zone diameters of

each sample. LR-AgNP/PLGA nanofibers have an average zone diameter of 12.5±0.3 mm in *E. coli* and *S. aureus* bacteria. PLGA nanofibers have been found to exert the lowest antibacterial effect among nanofibers by forming 8.5±0.2 mm zone diameter on *E. coli* bacteria. In another study, the antibacterial effects of licorice root on *E. coli* and *S. aureus* bacteria were investigated and the mean zone diameters were calculated as 13±0.0 mm for *E. coli* and 14.4±0.4 mm for *S. aureus* (Sedighinia et al., 2012). In another similar study by Shahid et al. (2022), PVA nanofiber scaffolds containing licorice root were produced. It was determined that these nanofiber scaffolds showed extraordinary antibacterial activity against *S. aureus* bacteria. It was determined that this result may be related to the antibacterial effect of licorice root. In our study, the mean zone diameters formed by LR-AgNP/PLGA nanofibers in *E. coli* and *S. aureus* bacteria were 12.5±0.3 mm. In addition, in our study, when the mean zone diameters were evaluated, it was determined that the samples with the highest antibacterial effect were LR-AgNP/PLGA nanofibers for both bacteria. We can say that licorice extract gives nanofiber a strong antibacterial property. Studies in the literature confirm the data obtained from our study that licorice root has antibacterial effect (Sedighinia et al., 2012; Varsha et al., 2013).

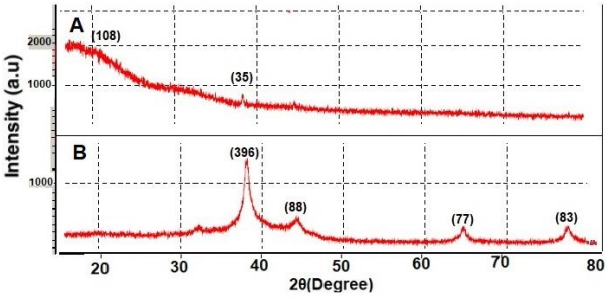

Figure 4. XRD plots **A)** LR-AgNP/PLGA, **B)** LR-AgNP.

The antioxidant capacity of licorice extract was analyzed by DPPH method, and it was calculated that the equivalent of ascorbic acid is 2.72 µM and the value of DPPH% removal is 1.2. The results showed that licorice root has antioxidant properties. In another study conducted with licorice root extracts in the literature, ascorbic acid and DPPH % removal values of licorice root extracts were calculated and it was concluded that licorice root has antioxidant capacity (Tohma & Gulçin, 2010). The results obtained from the literature and our study support each other and reveal that licorice root has antioxidant properties in terms of antioxidant and bioactive compound concentration (Lateef et al., 2012).

Wound healing processes of six different groups were evaluated in the scratch assay. For each group, three replicate experiments were performed, and *in vitro* wound scratch models were created. The wound closure process was observed for 48 h by imaging every 3 h starting from the 0th h. Microscope images taken at 0 and 12 h for each of the six groups are given in

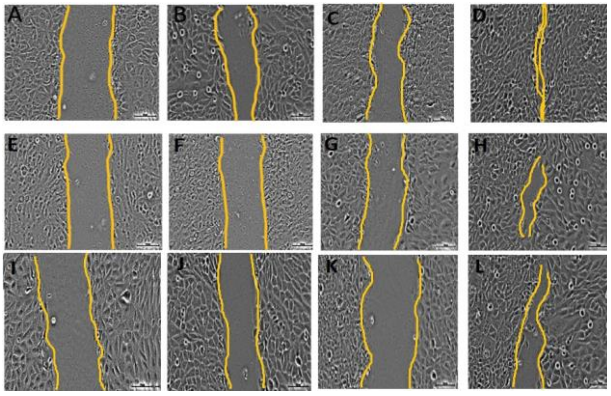

Figure 5. Microscope images illustrating the wound healing progression in all groups at 0 and 12 h. **A)** Control group at 0 h, **B)** Control group at 12 h, **C)** 1% LR-AgNP/PLGA group at 0 h, **D)** 1% LR-AgNP/PLGA group at 12 h, **E)** PLGA group at 0 h, **F)** PLGA group at 12 h, **G)** 2% LR-AgNP/PLGA group at 0 h, **H)** 2% LR-AgNP/PLGA group at 12 h, **I)** AgNP/PLGA group at 0 h, **J)** AgNP/PLGA group at 12 h, **K)** 3% LR-AgNP/PLGA group at 0 h, **L)** 3% LR-AgNP/PLGA group at 12 h.

percentages of each group were determined using the wound healing percentage formula. Figure 5. The images were analyzed using ImageJ software and scratch areas were calculated. As a result of these calculations, the wound closure The fastest wound closure was observed in the group of 1% LR-AgNP/PLGA nanofibers. Statistical analysis revealed that the group using 1% LR-AgNP/PLGA was statistically significantly different from the control and AgNP/PLGA groups, indicating that 1% LR-AgNP/PLGA is effective in accelerating wound healing (Figure 6).

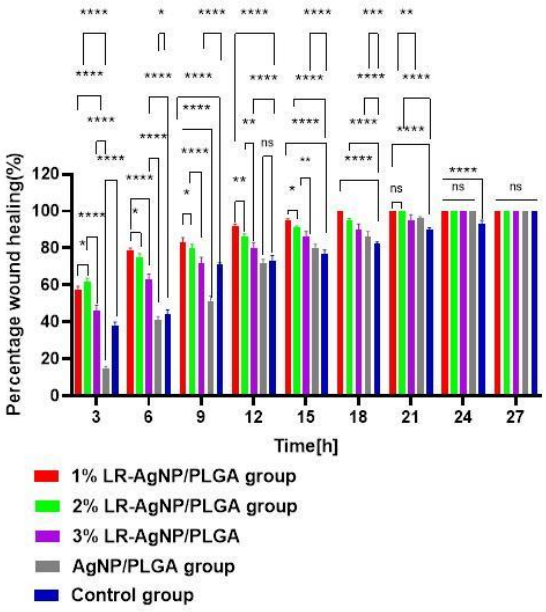

Figure 6. Percentage wound healing graph of all groups. *(**** P <0.0001, ** P <0.01, *** P <0.001, *P <0.05, ns P>0,05).*

Roy et al. (2023), determined that licorice root extract did not have a cytotoxic effect on cells and that the group containing licorice root extract increased cell healing when compared the healing process of the cell line with controlled cell lines in an *in vitro* scratch model.

Siriwattanasatorn et al. (2020) determined that licorice plant extracts have wound healing abilities *in vitro*. Zabihi et al. (2023) conducted an in vivo study by testing hydrogels prepared with licorice root extract on burn patients and determined that hydrogels containing licorice root significantly accelerated wound healing compared to the control group.

In this study, the fastest wound healing in the *in vitro* wound scratch model was realized in the 1% LR-AgNP/PLGA group, and it was observed that it increased the wound healing by 18% compared to the control group. As a result, it was determined that licorice root has a wound healing effect and significantly accelerates wound closure compared to the control group and our findings are in parallel with the studies in the literature.

Conclusion

LR-AgNPs were synthesized by green synthesis method with the medicinal plant licorice root, and these synthesized LR-AgNPs were used in the production of nanofiber wound dressings by electrospinning method with PLGA. *In vitro* cell culture studies were performed along with characterization tests of nanofibers after production.

The antioxidant capacity of the nanofibers obtained; it was determined that the antioxidant properties of licorice root were higher than nanofibers. All the nanofibers showed antibacterial properties by creating an inhibition diameter against *S. aureus* and *E. coli* bacteria. By examining SEM images of nanofibers, nanofibers with an average fiber diameter between 260 nm- 357 nm were obtained in a smooth continuous cylindrical structure. The crystal structures of the materials were analyzed by XRD analysis. The chemical structures of the nanofibers were examined by FTIR analysis and the FTIR graphs were found to be similar to the characteristic peaks of PLGA. In addition, 2% LR-AgNP-PLGA and 3% LR-AgNP-PLGA nanofibers were produced in order to examine the effect of the increase in licorice amount on cell healing in the wound scratch model *in vitro*. Increasing the amount of licorice did not have a positive effect on the wound closure rate, and it was determined that the fastest wound healing among all nanofibers produced was in the 1% LR-AgNP-PLGA nanofiber. As a result of these findings, silver nanoparticle nanofiber wound covers containing licorice extract can be used in wound healing studies and have the potential to be an effective wound dressing.

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Author Contributions

AM: Conceptualization, Project Administration, Writing – Review & Editing; MES: Data Curation, Formal Analysis, Investigation, Visualization and Writing – Original Draft Preparation; BS: Investigation, Resources, Writing – Review & Editing; ZGM: Supervision, Project Administration, Validation, Writing – Review & Editing.

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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