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Original Article

Effectiveness of Alginate Hydrogel Containing Ganoderma Polysaccharides on Wound Healing in Rat Model

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Abstract

Introduction: Infectious wounds are one of the most important problems of today's society, which lead to a delay in the stages of wound healing. *Pseudomonas aeruginosa* is one of the most common infections in trauma wounds. Ganoderma is an important mushroom with bioactive compounds that are widely used in traditional medicine on *P. aeruginosa*. This study aimed to assess this mushroom's effectiveness and antimicrobial properties on *P. aeruginosa*.

Methods: The polysaccharide compound was extracted from the *Ganoderma lucidum* mushroom by the Soxhlet method and then purified. The determination of concentration and characterization was done by the FTIR method. Then, the effect of polysaccharide on *P. aeruginosa* ATCC 9027 was evaluated on MIC and MBC by broth dilution tests. Next, alginate/collagen polysaccharide hydrogel was synthesized by thermal method, and the toxicity was evaluated on the L929 mouse fibroblast cell line. Fifteen male Wistar rats were randomly divided into control, positive control, and Al/col/Ps. Histopathological changes were studied. Data were analyzed using SPSS 26 and non-parametric Kruskal-Wallis and Mann-Whitney tests.

Results: MIC and MBC concentrations of the polysaccharide compound against *Pseudomonas aeruginosa* were calculated as 1.56 and 3.13 mg/ml, respectively. In this study, the sample cell viability with an acceptable survival rate of > 70% (toxicity < 30-35%) was the same as the control, indicating non-toxicity compared to the control sample. According to wound histopathology, wound closure (86%), fibroblast 18520, hair follicle 4, angiogenesis, collagen, and epidermis were significantly higher in the Al/col/Ps system group compared to the control group.

Conclusion: Besides antimicrobial activity against the standard *P. aeruginosa*, the extracted polysaccharide plays a significant role in infectious, traumatic wounds. Our results demonstrate that the Al/col/Ps hydrogel system can be recommended as a potential treatment for trauma wound infections.

Keywords: Polysaccharide, *Pseudomonas aeruginosa*, Wound Healing.

Introduction

A traumatic injury refers to physical injuries of sudden wound onset and severity, which is a major problem of people referring to emergencies and in the battle arena. These injuries mainly range from slight scratches and cuts to wounds with broad tissue injury and damage to bones and internal organs [1]. The post-occurrence signs of traumatic wounds and injuries appear as swelling, inflammation, hemorrhage, infection, and discharge from the wound site. Thus, controlling infection as a crucial cause of traumatic injuries can accelerate traumatic wound healing. Among wounds, problems in infectious diabetic foot ulcers are mainly caused by infection, which can be controlled to achieve an effective and new formulation to control traumatic infection [2, 3].

Among infectious agents in patients suffering from diabetic foot ulcers, Gram-negative bacteria play the most significant role. Owing to its unique physiological features, *Pseudomonas aeruginosa* is a major microbial cause of diabetes-related infections, particularly in developing countries, including Iran [4].

P. aeruginosa produces many metabolites or exogenous biological products, including elastase, alkaline protease, hemolysin, exotoxin A, and exoenzyme S, which, together with their heterogeneous lipopolysaccharides, are the main pathogenic agents. Many of these agents are involved in wound infection development [5].

Using compounds extracted from mushrooms with very high antimicrobial properties and low toxicity is particularly interesting in wound dressing to control wound infection. Among these, the Ganoderma mushroom contains high polysaccharide levels with antimicrobial properties, which has received considerable attention from researchers in recent years [6].

Ganoderma polysaccharides show a wide range of bioactivities, including anti-inflammatory, hypoglycemic, anti-ulcer, antitumor, and immune system stimulant effects. Polysaccharides' antitumor and anticancer effects are generally based on improving the host's immune system instead of direct cytocidal effects. Their secretory products also affect different immune system elements, including active macrophages, NL cells, and cytotoxic T cells [7].

G. lucidum polysaccharide compound can be used against pathogenic agents, mainly bacteria. The focus on the anti-chromium-sensing anti-pathogenic properties of mushrooms can introduce them as a new method for treating various infections, including antibiotic-resistant microbial strains [6].

Polysaccharide compound is typically isolated from a fermentation medium, mycelium, or fruiting body. The main factor for the fruiting body formation is the temperature in the optimum range of 25-30 °C [8]. This compound prevents the growth of a wide range of microorganisms and helps improve the wound's stages. [6]. In case of skin damage, it is necessary to cover the wound with a dressing quickly. Maintaining a moist environment in the wound, creating ventilation, preventing the transmission of microorganisms, and removing wound secretions are among the characteristics of an ideal dressing [2]. Finding dressings that do not have many side effects, are of natural origin, and processing is cheap and accessible, and also accelerate wound healing by creating a suitable bed. Among the recent dressings that have received attention are hydrogel dressings of natural origin [9]. This research used sodium alginate and collagen as promising candidates for wound treatment.

Alginate is an anionic polysaccharide that can serve as a hydrogel in normal conditions at room temperature. Owing to its high biocompatibility, low toxicity, and excellent gelling properties, this compound is commercially prepared from brown algae [2] and through the biosynthesis of Azotobacter. Alginate dressings fall into the type 3 dressing, that is, those with biological activities. The specific and unique properties of alginate dressings include non-toxicity, non-carcinogenicity, non-allergenicity, high absorption, hemostatic, strength agent, biocompatibility, sterility, manipulation ability, and low-cost processing technology [2,10].

Collagen consists of three related polypeptides forming a triple helical structure. Collagen diversity results from differences like their constituent polypeptides. Collagen fibers are formed by the self-assembly of collagen helices, with the ends of adjacent molecules overlapping, which causes high stretchability of collagen fibers [11]. Collagen fibrils provide the major biomechanical framework for cell attachment and anchoring of macromolecules, determining the tissue shape and maintenance. Collagen peptides have a high water-holding capacity and anti-radical activity [12].

This research aimed to investigate the antimicrobial and healing effect of polysaccharides extracted from Ganoderma Lucidium mushroom to use alginate dressings to accelerate the healing of skin wounds in rats.

Methods

Cultivation of Ganoderma lucidum mushroom

The *G. lucidum* strain was obtained from the mycology collection center of the Pest Control Organization, Agriculture Jihad Organization of Iran. The obtained strain was first revived in a potato dextrose agar culture medium. The cultured plates were incubated at 30 °C for ten days. After growth observation, several culture passages were prepared for preservation and incubated in the conditions above. Growth-containing plates were refrigerated at four °C for subsequent tests [13].

Production of mushroom fruiting body

The mushroom fruiting body was prepared using a freshly cultured plate of *G. lucidum*. Then, husk wheat germs were poured into flasks and sterilized at 121 °C for 15 min. Grown plates were divided into small pieces

under sterile conditions and poured into the flasks, which were incubated at 30 °C for one week. Finally, grown fungal germs were poured into bags containing a mixture of sawdust, bagasse, wheat bran, salt, and water. Mushroom-inoculated bags were transferred to an incubator for hyphae formation and the growth phase initiation. The hyphae formation process and fruiting in *G. lucidum* are completed in about 10-12 weeks [12].

Polysaccharide extraction processes by the Soxhlet method

Dried *G. lucidum* fruiting body powder (10 g) was fully dissolved and extracted with 200 ml of 80% ethanol using a Soxhlet (Fat Extractor, Iran) apparatus. To this aim, the mushroom powder was filled inside the cylinder and inserted in the main extraction chamber. This process was performed during six cycles using 80% ethanol at 80 °C for nine h. After extraction, the solvent was removed using a rotary (RE 100, Iran) evaporator, and the mushroom powder was discarded from the cylinder. The extracted solvent was poured into a vessel and dried at room temperature [14].

Polysaccharide purification process

Polysaccharides and high amounts of protein and lipids were extracted in the extraction method. Extracted polysaccharides were purified by removing proteins and lipids using the Sevage deproteinization method and defatting using organic solvents.

Sevage deproteinization

In this method, 10 ml of the aqueous polysaccharide was transferred to a glass tube. Then, a three-fold chloroform-butanol solution (in a 1:4 ratio) was added to this solution. The obtained solution was vigorously shaken manually for 5 min and then placed in a fixed environment to reach equilibrium for 15 min. Two phases were formed after the equilibrium, and the polysaccharide-containing phase was separated afterward [9].

Defatting using organic solvents

The previously dried polysaccharide samples were used for this procedure. First, 20 ml of distilled water and 50 ml of hexane solvent were poured into a falcon tube, which was shaken several times to create two phases. The supernatant containing hexane-dissolved fats was separated, and the lower phase containing distilled water-dissolved polysaccharide was poured into another falcon tube. Then, 20 ml of chloroform was added to the polysaccharide-containing falcon and shaken to create two phases. The lower phase containing water-soluble polysaccharides was transferred to another falcon tube and received 20 ml of ethyl acetate. The tube was shaken to create two phases, and the supernatant was discarded to obtain the lower phase containing purified polysaccharide [15].

Quantitative and qualitative identification of extracted polysaccharide

The presence or absence of polysaccharides was identified qualitatively using the Molisch test. The concentration of extracted polysaccharides was measured using a standard phenol-sulfuric acid curve. To make Molish's reagent, 3.75 grams of alpha-naphthol was first dissolved in 20 ml of ethanol. Then, in a test tube, 2 ml of the solution containing the extracted polysaccharide was poured, and a drop of alpha-naphthol solution was added and mixed. Then 3 ml of concentrated sulfuric acid solution was slowly added to the solution through the wall of the test tube [16].

Quantification of extracted polysaccharide

This procedure was based on the phenol-sulfuric method, in which a standard curve is drawn to measure an unknown solution's optical density (OD). A known amount of the extracted polysaccharide extract was measured with a balance and dissolved in 1 ml of water to quantify extracted polysaccharide. Then, 200 µl of polysaccharide extracts were poured into a screw cap test tube, into which 1 ml and 0.2 ml of A and B reagents were respectively added precisely so that the reagents were not in contact with the test tube wall. The samples were then exposed to 30 °C for 20 min, after which the absorbance of samples was read against a blank (distilled water) at 490 nm using a spectrophotometer (Shimadzu, Japan) [16].

Polysaccharide extract characterization with FTIR spectroscopy

The powder sample for FTIR (Tensor, Germany) was sent to Poyan Exir Technology Company, and its absorbance was measured at 400-4000 cm−1 wavelengths [17].

Antibacterial effects of the polysaccharide extract

The lyophilized standard *P. aeruginosa* strain ATCC 9027 was obtained from the National Research Center

for Genetic Engineering. The strain was cultured in a Mueller-Hinton agar (MHA) medium.

Sensitivity determination tests on P. aeruginosa

The sensitivity of *P. aeruginosa* to polysaccharides was evaluated using the well diffusion test, the MIC, and MBC.

Sensitivity determination of G. lucidum polysaccharide

Initially, 0.05 g of dried polysaccharide composition powder was completely dissolved in 1 ml of distilled water. The prepared polysaccharide composition (100 µl) was added to these wells and incubated at 37 °C for 24 h, followed by measuring the growth inhibition zone diameter [18].

Determination of MIC and MBC

Polysaccharide was diluted with an initial concentration of 50 mg/ml. Next, serial dilutions (25-0.045 mg/ml) were prepared for the polysaccharide composition.

Toxicity evaluation of G. lucidum polysaccharide

To obtain a criterion of the biocompatibility of samples, the proliferation of cells cultured on the samples was studied using the MTT assay on the murine fibroblast cell line for 24 h. Polysaccharides (Ps) were used at concentrations of 0.78, 1.56, 3.13, and 6.25 mg/ml [19].

Synthesis of a polysaccharide-containing alginate/collagen hydrogel (Al/col/Ps)

According to Geo et al.'s method, to make alginate/collagen hydrogel, first, dissolve 2% w/w sodium alginate solution in distilled water at high temperature by magnetic stirrer and then add 1% w/w collagen solution in equal proportion at a lower temperature is added to the mixture. Next, a calcium carbonate suspension was mixed into the sodium alginate and collagen solutions. Finally, the Al/col was obtained after the reaction for 15 min. Polysaccharides at the concentrations determined after MIC and toxicity measurements were then refrigerated in sterile 50 ml falcons at four °C [20].

The method of creating a skin wound and creating an experimental infection in the wound

Before wounding, male rats were anesthetized by intraperitoneal injection of ketamine (30 mg/kg) and xylazine (4 mg/kg). To create a wound, after removing the hair from the back of the neck, three circles with a diameter of 8 mm were created on the back of the mice with a punch. Moreover, the day of surgery (wound creation) was considered a zero-day [21]. Then, in order to infect the wounds, 108 CFU of freshly cultured Pseudomonas aeruginosa bacteria were inoculated in a volume of 30 micrograms for each wound [22].

Animal treatment method

All treatments were performed three days after the wound infection. According to the previously mentioned grouping, the control group received no treatment for the wounds of rats infected with the *P. aeruginosa* strain. The following are treatments (a final volume of 50 µl per wound) for infectious wounds of test groups twice daily for 21 days.

1) Control group: infectious wounds with no drug treatment (negative control)

2) Blank group: sulfadiazine ointment at 20 mg/ml (positive control)

3) Ps – AL/Col group: infectious wounds treated with alginate/collagen and polysaccharides at 2 and 3.13 mg/ml, respectively.

Sampling and macroscopic and microscopic examination

Photographs were taken of all wounds of all groups on days 4, 7, 14, and 21. The area of each wound was determined using ImageJ 1.52i software. To check the microscopic sections, the cut area and the samples, after washing in physiological serum solution, were transferred to a container containing 10% formalin fixative after performing the passage steps. Tissue for dehydration and toluene for tissue clarification, paraffin molds were transferred to a microtome device. Sections of 5-7 microns were prepared and stained with hematoxylin and eosin methods. The samples were evaluated using a photomicroscope in the microscopic observations of the sections. Histologically, the number of inflammatory cells in the wound healing area in all groups was evaluated with 400 magnifications. In all stages, the ethical rules and regulations of working with animals were observed [21].

Analysis of data

Data were analyzed using SPSS 26 software. Groups were compared with non-parametric Kruskal-Wallis and Mann-Whitney tests at a statistical significance of

$P < 0.05$. The significance level and graphs were represented by GraphPad Prism 8 software.

Results

Polysaccharide extraction and purification by the Soxhlet method

An extraction efficiency of 23% was determined according to the formula.

Quantitative and qualitative identification of extracted polysaccharide

The polysaccharides extracted from *G. lucidum* were identified qualitatively and quantitatively using the Molisch test and drawing a standard phenol-sulfuric acid curve. According to the results obtained from the Molisch test, the violet halo indicates the presence of polysaccharides in the extracted solution.

Quantification of extracted polysaccharide

The extracted polysaccharide was quantified by drawing a standard curve using glucose and phenol/sulfuric acid reagents. The OD of samples was then read at 490 nm. The extracted polysaccharide was quantified using the following linear formula obtained from this standard curve. In this formula, y and x represent the unknown solution OD and the extracted polysaccharide concentration, respectively. This formula indicates a polysaccharide concentration of 34%, showing the highest value.

Polysaccharide characterization with FTIR spectroscopy

Figure 1 shows the characterization results of polysaccharides extracted from *G. lucidum* using FTIR spectroscopy. The first peak at 616 cm−1 belongs to the carbonyl group with a double bond. The second peak around 1049 cm−1 represents CO stretching bands. Another peak around 3326 cm−1 results from the alkane group's O-H bond stretching vibration, suggesting the presence of hydroxyl groups in polysaccharides. The absorbance of *G. lucidum* polysaccharides peaked at 3326 and 1636 cm−1, characteristic of glucopyranose and beta-glucan, indicating that *G. lucidum* polysaccharides have a beta-glucan configuration as observed in the FTIR spectrum.

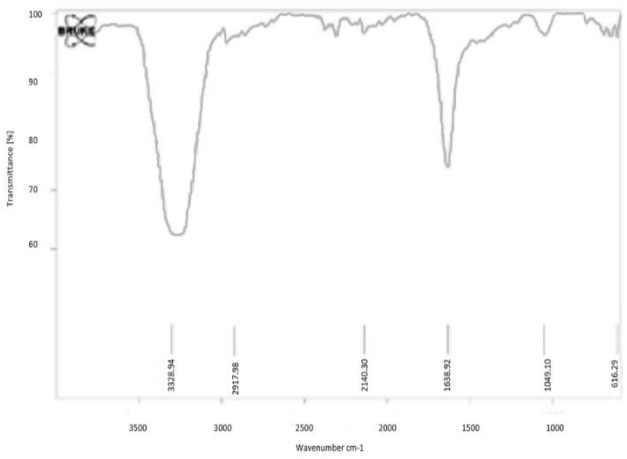

Figure 1: The FTIR spectrum of polysaccharide extract at 400-4000 cm^{-1} wavelengths.

Sensitivity determination with the well diffusion test

The observation and measurement of the inhibition zone around the well confirmed the antimicrobial activity of the produced polysaccharide. As shown in Figure 2, the inhibition zone is 18 mm for the standard strain by the G. lucidum polysaccharide, but no inhibition zone is observed in the blank well. This result demonstrates the antimicrobial activity of polysaccharide on the P. aeruginosa.

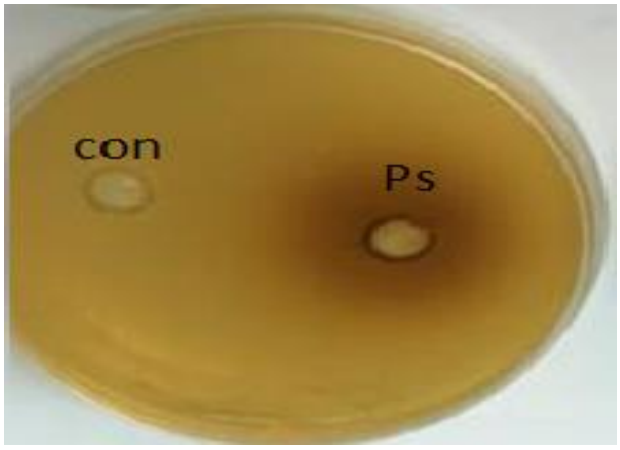

Figure 2: Well diffusion test of Ganoderma polysaccharide composition against standard Pseudomonas aeruginosa**.** The diameter of the non-growth halo measured for the standard strain by Ganoderma polysaccharide is 18 mm, but no halo is observed in the control well.

Determination of MIC

The antimicrobial activity of Ganoderma polysaccharide diluted in agar against standard *Pseudomonas aeruginosa* was determined as 1.56 mg/ml.

Determination of MBC

The antimicrobial activity of Ganoderma polysaccharide diluted in agar against standard *Pseudomonas aeruginosa* was determined as 3.31 mg/ml.

Toxicity of polysaccharide and Al/col/Ps on the cell line

Figure 3 depicts the survival rate of the L929 murine fibroblast cell line after 24 h of exposure to four concentrations of the polysaccharide MIC with the replications ($P \leq 0.05$). In this test, all samples showed cell viability with an acceptable survival rate of > 70% (toxicity < 30-35%), the same as the control, indicating non-toxicity compared to the control sample. Among the four MIC values, the highest polysaccharide concentration of 3.13 (78%) was used as the acceptable survival rate in the animal phase.

Data were analyzed using SPSS 26 software at a statistical significance of $P < 0.05$. The standard deviation (SD) and graphs were represented by GraphPad Prism 8 software (Fig. 3).

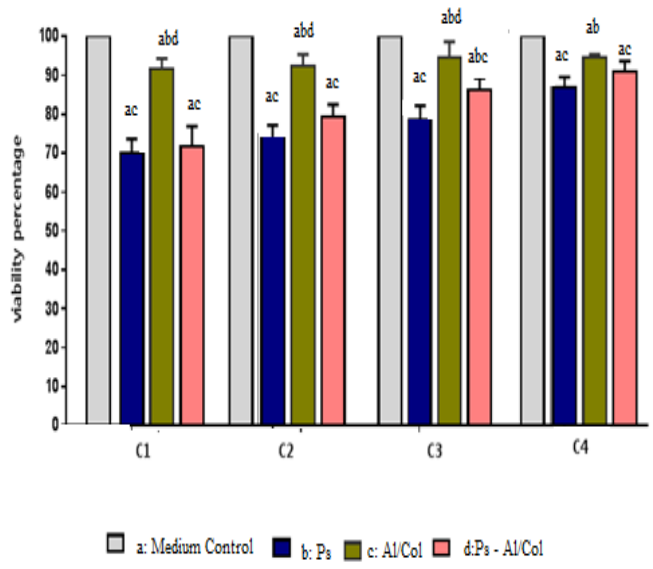

Figure 3: The survival rate of the L929 murine fibroblast cell line exposed to the polysaccharide, the alginate/collagen hydrogel, and the hybrid polysaccharide-alginate/collagen hydrogel system at four concentrations of C1-C4. The concentration of C2, which included the concentration of polysaccharide (3.13 mg/ml equal to 78%), was used as the acceptable survival rate in the animal phase.

Macroscopic examination of the wound healing process

In the macroscopic examination, the wound closure rate was calculated on 0, 4, 7, and 14 days after measuring the wound area using Image J software and determining wound size reduction relative to the initial wound in the same group. The control and other groups had three and five replications, respectively. Using the Mann-Whitney test, the signs above the columns show significance levels between treatment and control groups. Data are represented based on mean ± SD.

The results of the wound recovery rate revealed that the wound area was the same in all three groups on the first day, but it decreased significantly in control compared to the other groups from the fourth day to the end of the study. This difference was low in the initial days, but closer differences were observed toward the last days. The wound area was significantly different among the groups treated with Al/col/Ps compared to the control group in the macroscopic examination. This difference was obviously seen on days 14 and 21, and wound closure and healing rates were higher than control and positive control groups. Figures 4 and 5 exemplify wound closure and healing rates, indicating the highest wound closure rate (about 76%) in the Al/col/Ps group on day 21.

Microscopic and histopathologic examination of wounds

The results showed that the highest wound healing rate belonged to the AL/Col-Ps treatment group compared to the negative and positive control groups. Wound healing rates increased gradually during different days and were maximized on day 21 compared to the other days (7 and 14). The results of hematoxylin/eosin (H & E) and Masson's trichrome staining revealed a considerable wound healing rate in the AL/Col-Ps treatment group. The results of angiogenesis, fibroblastosis, hair follicle growth, epidermis, collagenesis, and inflammation obtained after H/E and Masson's trichrome tissue staining are separately shown in Figure 6. The results of the Mann-Whitney statistical analysis indicated a significant wound healing rate in the AL/Col-Ps treatment group compared to the control and positive control groups on days 7, 14, and 21.

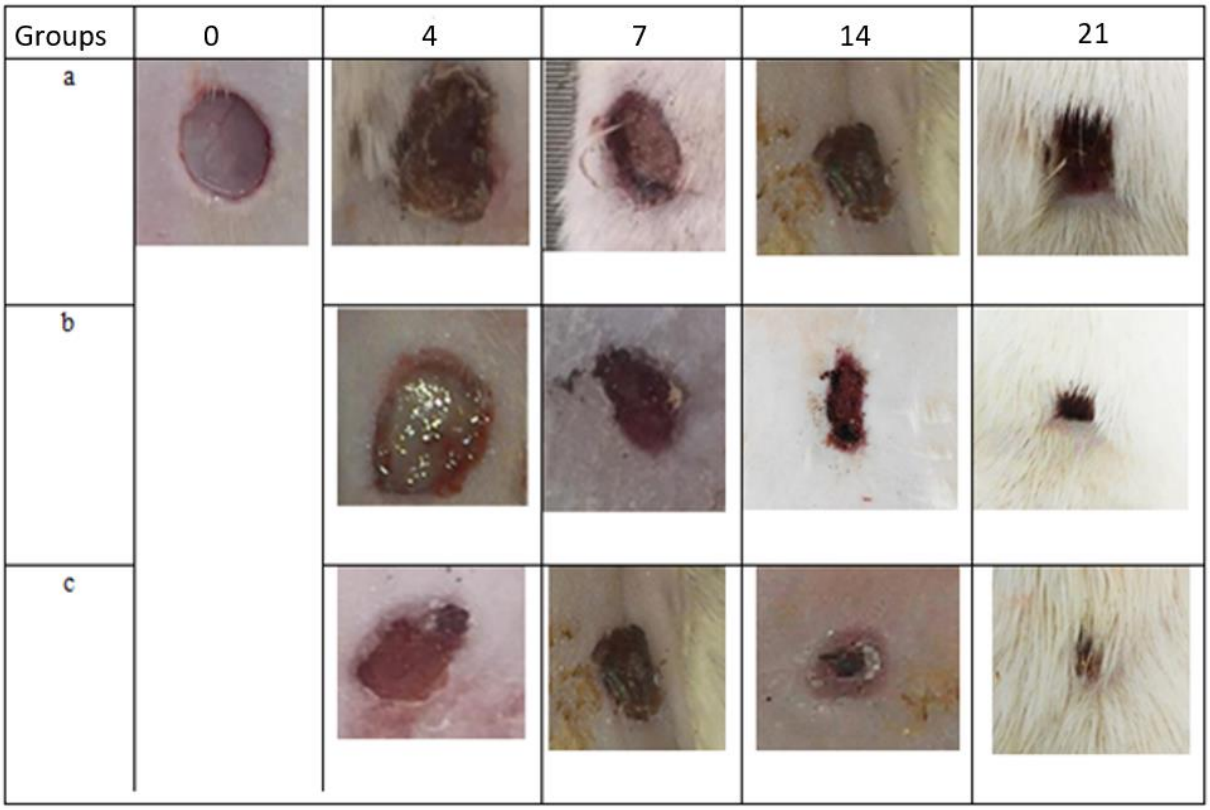

Figure 4: Total Average Healing Time. Wound closure and healing processes in the control (a), sulfadiazine (b), and AL/Col-Ps groups on days 0, 4, 14, and 21. The results are presented as a The concentration of polysaccharide used is equal to 3.13 mg/ml

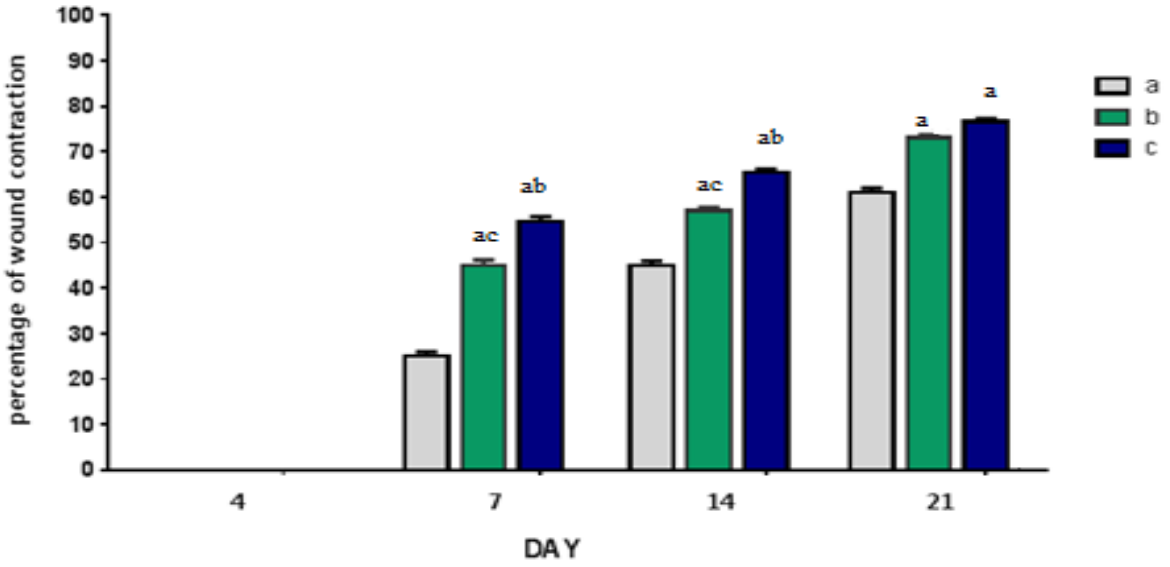

Figure 5: Wound healing rates in the control (a), sulfadiazine (b), and AL/Col-Ps (c) groups. The signs above the columns show significance levels between treatment and control groups using the Mann-Whitney test.

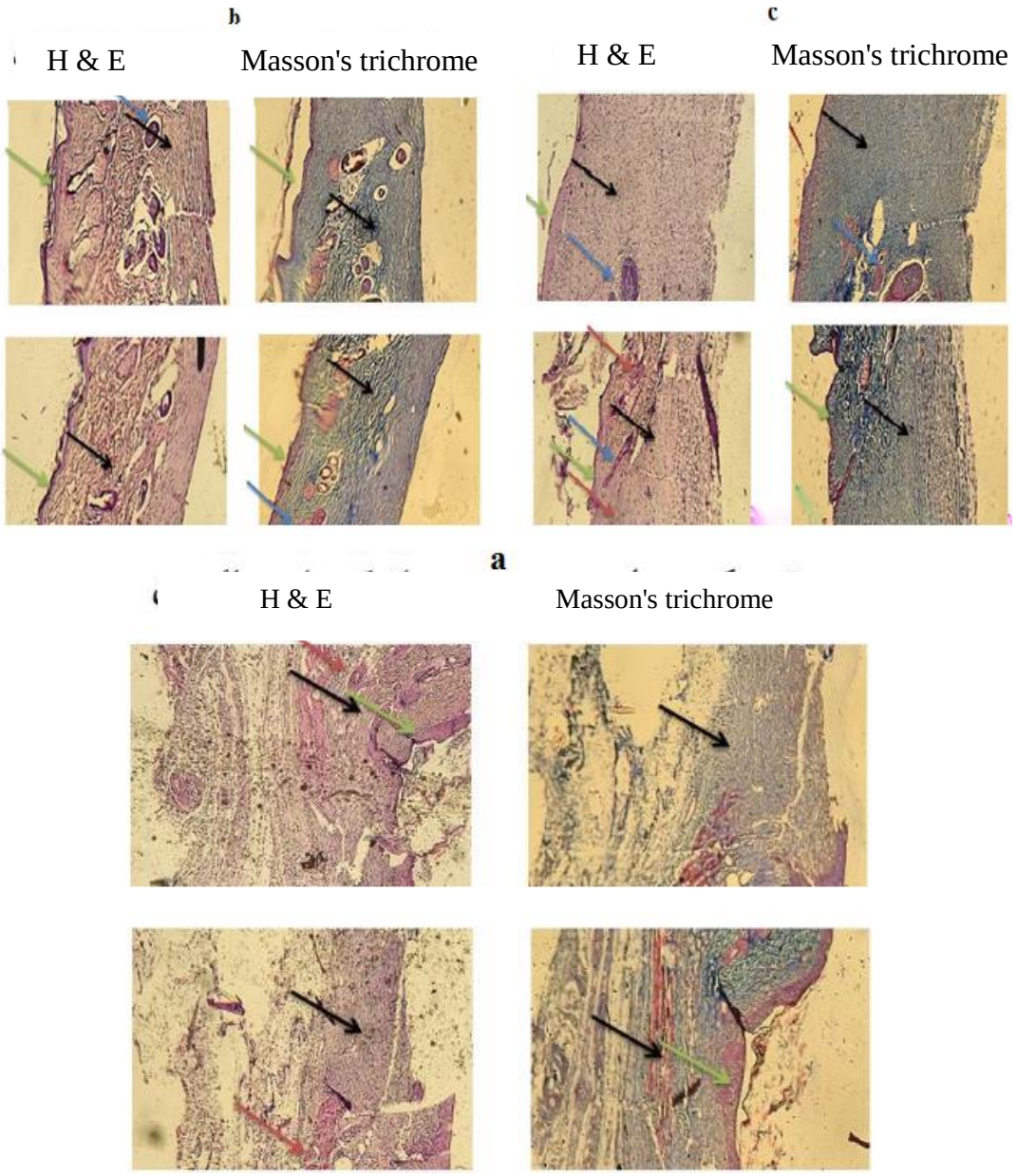

Figure 6:. Results of H/E and Masson's trichrome tissue staining (×40) on day 21. Black arrow: fibroblast, blue arrow: hair follicle, red arrow: blood vessels, green arrow: epidermis.

Density of angiogenesis

Average blood vessel densities in the drug and positive control groups were not significantly different from the control on day 4. On days 7 and 14, average blood vessels increased in all treatment groups, significantly different from the control ($P \leq 0.05$). The maximum angiogenesis rate in all treatment groups was observed on day 7. The AL/Col-Ps group recorded the highest angiogenesis rate, significantly different from the control and positive control groups. Vessel density decreased from day 14, and the AL/Col-Ps and positive control groups significantly differed from the control group ($P \geq 0.05$; Fig. 7).

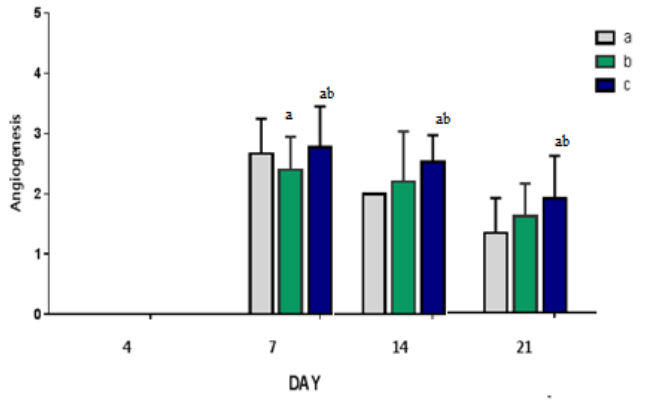

Figure 7: Comparison of the numerical density of blood vessels in different groups on days 7, 14, and 21. Using the Mann-Whitney test, the signs above the columns show the significance level ($P \leq 0.05$) between treatment and control groups.

Number Fibroblasts

The number of fibroblasts significantly differed in the experimental groups on days 7 and 14, but no significant differences were observed on day 21. The number of fibroblasts increased in the AL/Col-Ps group (18,520), with a significant rise in this group on day 14 (Fig. 8).

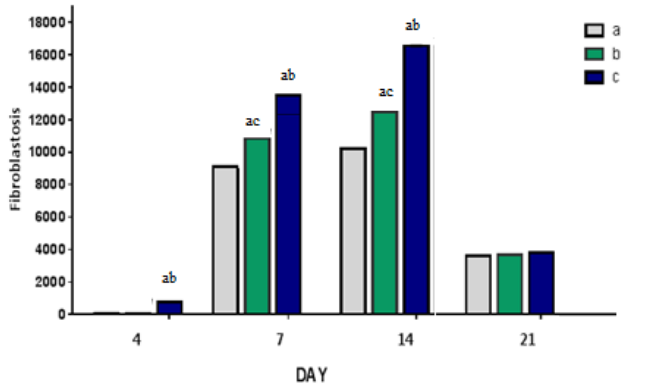

Figure 8: Statistical comparison of the number of fibroblasts in different groups on days 7, 14 and 21. Upper signs ($P \leq 0.05$) indicate significance between the treated groups and the control group, which was performed with the Mann-Whitney test.

The results of hair follicle growth investigation

One of the indicators of the quality of healing at the wound site is the presence and regrowth of hair follicles. According to the obtained results, no follicles were observed on the 7th and 14th days, but on the 21st day, we saw the growth of hair follicles in the samples. Hence, the average number of follicles observed in control, positive control, alginate/collagen hydrogel with polysaccharide groups was 1, 2, 3 (picture 9).

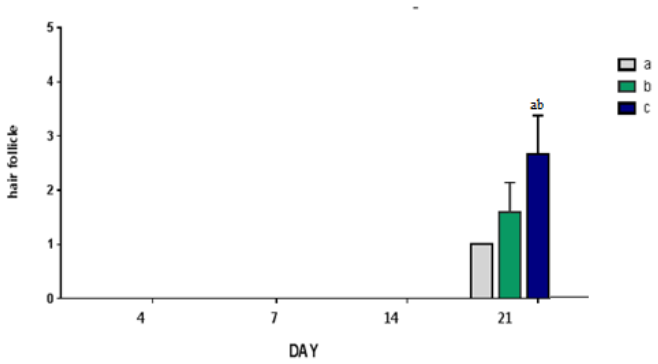

Figure 9: Statistical comparison of hair follicle density in different groups on days 7, 14, and 21. The signs above the columns show the significance level ($P \leq 0.05$) between treatment and control groups using the Mann-Whitney test.

Results of epidermis growth

An effective strategy to determine the wound recovery rate is to measure the epidermis formed at the wound site, which is expressed as a percentage compared to the adjacent healthy skin tissue. The epidermis examination results indicated that the number of epidermal cell layers was lower than the other groups on day 7, and the epidermis level increased gradually during 21 days. The highest epidermis growth was observed in the AL/Col-Ps treatment group (Fig.10).

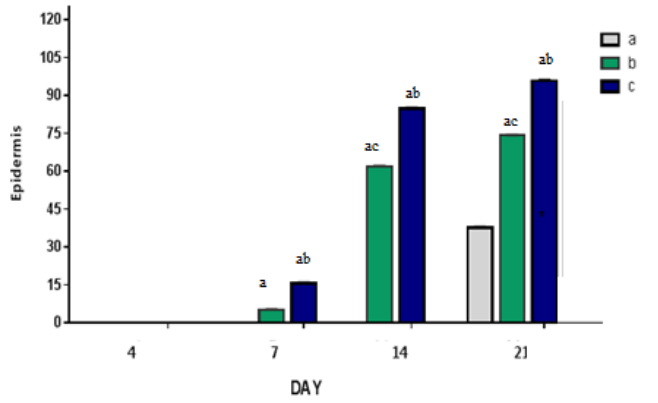

Figure 10: Epidermis thickness in different groups on days 7, 14, and 21. The signs above the columns show the significance level ($P \leq 0.05$) between treatment and control groups using the Mann-Whitney test.

Results of collagenesis

According to the collagen measurements, the experimental samples significantly differed in this parameter on days 7 and 14. The highest growth and presence of collagen fibers in the tissue were recorded in the AL/Col-Ps group on days 14 and 21 (Fig. 11).

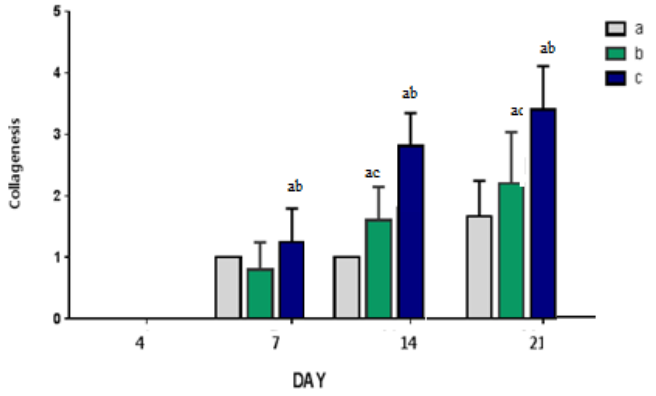

Figure 11: Collagen fiber densities in different groups on days 7, 14, and 21. The signs above the columns show the significance level ($P \leq 0.05$) between treatment and control groups using the Mann-Whitney test.

Results of inflammation

The results for the inflammation index revealed an increase in the average number of inflammatory cells in both groups after wound formation in the initial days compared to the control group. On day 7, the number of inflammatory cells was uppermost in the positive control and control groups compared to the lowest inflammation rate detected in the AL/Col-Ps group. The same group showed the lowest inflammation rate on day 21 (Fig. 12).

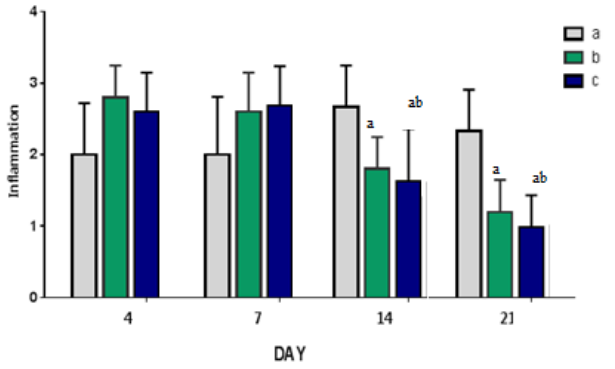

Figure 12: Comparison of inflammation rates in different groups on days 7, 14, and 21. The signs above the columns show the significance level ($P \leq 0.05$) between treatment and control groups using the Mann-Whitney test.

Discussion

Wound healing is a complex process that includes various molecular processes such as inflammation, angiogenesis, re-epithelialization and wound closure [23]. Ganoderma lucidum mushrooms contain specific bioactive compounds that are considered due to their efficiency and absence of side effects [24]. Ei et al. [25] reported that numerous polysaccharides were extracted from *G. lucidum* using various techniques and solvents, with beta-1,3/1,6-glucans being the most common polysaccharide composed of various combinations of sugars with different molecular weights. Yang [26] qualitatively analyzed and evaluated bioactive compounds extracted from *G. lucidum*; in the tests of their study, methanol and ethanol yielded extracts with the highest efficiency of bioactive compounds. The bioactive compounds in the ethanolic extract obtained from *G. lucidum* were evaluated quantitatively, and the researchers concluded that ethanolic extract obtained from *G. lucidum* showed the highest inhibitory effect against the tested pathogenic agent regarding the antibacterial property. According to Kowan, the most active components are not generally water-soluble. Hence it is expected to obtain the most active extracts from less-polar organic solvents [27]. In the present study, polysaccharides were also isolated using ethanol, and *G. lucidum* ethanolic extract showed antibacterial activity by the well diffusion method.

Among extraction techniques, the Soxhlet method is more common due to easy access and ease of use based on a study by Saltarly [28]. Saavedra et al. [29] employed a Soxhlet apparatus for extraction from *G. lucidum*. They concluded that a hexane solvent is used in this device for continuous fungal powder extraction, which saves the solvent extraction efficiency and results in a high yield. They reported an extraction efficiency of 15% for ethanolic extract. In our study, polysaccharide extracts were isolated by the Soxhlet method, and the extraction efficiency was 34% after polysaccharide purification.

The polysaccharide structure was assessed by FTIR spectrometry. Shah observed that *G. lucidum* polysaccharides showed peak absorption at 3383 cm−1 due to the stretching vibration of the O-H bond, indicating the presence of hydroxyl groups in polysaccharides. Other bands at 2932 and 1630 cm−1 were stretching vibrations of the C-H bond and water. The peak appeared close to 1402 cm−1, resulting from the CO stretching vibration. The peak absorption at 1038 cm−1 was caused by the asymmetric stretching vibration of the COO bond on the polysaccharide ring, representing polysaccharides. A distinct peak of dextran also appeared in the spectrum. Moreover, the 894.8 cm−1 is a characteristic absorption peak of the

glucopyranose and beta-glucan relationship, suggesting the beta-glucan configuration in *G. lucidum* polysaccharides [30].

In comparison, our results also indicated that the absorption of *G. lucidum* polysaccharides peaked at 3326 and 1636 cm−1. It can generally be suggested that the extracted ethanolic extract contained polysaccharides, probably due to functional groups in the FTIR spectrum. Besides, 1636 cm−1 shows a characteristic absorption peak of the glucopyranose and beta-glucan association, implying that the *G. lucidum* polysaccharides have the beta-glucan configuration observed in the FTIR spectrum.

The antibacterial property of *G. lucidum* polysaccharides was investigated in various studies. Yun et al. [31] studied the direct antimicrobial effect of *G. lucidum* aqueous extract alone and in combination with four antibiotics on 15 bacterial species. The results revealed that *G. lucidum* was more effective than antimicrobial antibiotics against different strains such as *Escherichia coli, Micrococcus lotus, Staphylococcus aureus, Bacillus cereus, Proteus vulgaris,* and *Salmonella typhimurium*. Qeyvareshi et al. [32] evaluated *G. lucidum* chloroform extract because of its antibacterial effect on Gram-positive (*Bacillus subtilis, S. aureus,* and *Enterococcus faecalis*) and Gram-negative (*E. coli* and *P. aeruginosa*) bacteria. According to the results, this extract could inhibit the growth of two Gram-positive (*S. aureus* and *B. subtilis*) and Gram-negative bacteria (*E. coli* and *P. aeruginosa*) at MIC values of 8 and 6 mg/ml, respectively. Chen et al. [33] determined the antibacterial activity of *G. lucidum* aqueous, acetonic, and methanolic extracts by the disk diffusion method. The GIZ of mycelium acetonic extract was 30 mm against *P. aeruginosa*. In a study by Keypour on the antimicrobial activity of different *G. lucidum* extracts using the agar gel diffusion technique, *G. lucidum* ethanolic extract showed antibacterial effects (with GIZ) against *S. aureus* (29 mm), *P. aeruginosa* (25 mm), and *Fusarium* fungus (25.5 mm). This extract exerted similar inhibitory effects against bacterial agents *E. coli* (16 mm), *Klebsiella pneumoniae* (13 mm), and *Salmonella typhi* (19 mm) [40]. Eskalia et al. [34] reported that *G. lucidum* polysaccharides, in which D-glucose was the main component, were highly involved in the growth inhibition of pathogenic bacteria. Polysaccharide species isolated from the fruiting body of *G. lucidum* showed the highest inhibitory ability against *Micrococcus luteus*. The antimicrobial activity using disk diffusion tests indicated that *G. lucidum* chloroform extract showed growth-inhibitory effects on *S. aureus* and *B. subtilis* with a MIC value of 8 mg/ml. In the well diffusion method of our study, a GIZ value of 18 mm and MIC/MBC values of 1.56 and 3.13 mg/ml were obtained for the polysaccharide extract.

Cell model systems have been important for many biomedical studies. An ideal wound dressing should not release toxic products nor produce unwanted reactions, which can be examined through cytotoxic tests in the laboratory. The polysaccharide toxicity level has been investigated on various cell lines in different studies, most of which have evaluated toxicity levels at acceptable ranges.

According to Berger et al. [35], the MTT assay on macrophages treated with different concentrations of *G. lucidum* polysaccharide extract revealed that all these doses significantly increased cell viability rates. However, the highest effect was observed for a 0.1 µg/ml dose. In the present research, the results of polysaccharide cytotoxicity on the murine L929 fibroblast cell line using the MTT assay indicated that the use of polysaccharides to kill *P. aeruginosa* caused no toxicity in the body.

Limited studies are available concerning the effect of Al/col/Ps on wound healing, the results of which suggest delayed wound recovery in rats. This delay, in turn, results from significant changes in the recovery extent and percentage in wounded rat's relative to healthy ones. The combined Al/col/Ps can accelerate wound recovery in rats.

In the macroscopic examination, a lower wound area and a significantly different wound recovery rate were observed in the polysaccharide treatment group compared to the control group. In the microscopic examination on day 4, inflammatory cell accumulation in the Al/col/Ps treatment group was similar to the other groups. On day 7, the angiogenesis rate was higher in the Al/col/Ps treatment group than in the control and blank groups. Briefly, the angiogenesis rate was more than the other two groups in the rest of the recovery process. Collagen formation was more regular, and the epithelial cell growth was more significant in the sulfadiazine and Al/col/Ps treatment groups than in the

control group. Nevertheless, the epithelial cell and fibroblast growth rates were higher in the Al/col/Ps treatment group than in the sulfadiazine group.

In this investigation, wound size and recovery rates were lower in the Al/col/Ps treatment group than the sulfadiazine-treated group on days 7, 14, and 21, with a significant difference between the two groups. In a previous study, creatinine, cholesterol, and liver enzymes decreased in wounded rats [36], and their recovery rate was similar to healthy rats, as observed in our study.

In a study by Zhang et al. on rats, the wound healing rate was faster in a group treated with sodium alginate and type 1 collagen loaded with umbilical mesenchymal stem cells than in the control group. This combination accelerated wound closure compared to the control group [37]. In this research, the recovery rate was higher in the diabetic treatment group than in the other groups on all study days, suggesting the remarkable effects of Al/col/Ps on eradicating factors delaying wound recovery in rats.

Aderibigbe and Buyana investigated skin wound healing in rats. They reported that the angiogenesis rate and the resulting granulated tissue were maximized in the animals around day 7, showing the normal process of increasing angiogenesis. The results revealed wound healing and an accelerated wound recovery rate [38], which corresponds to our study. Consequently, the presence of polysaccharides led to a better angiogenesis rate than the control group (on the whole 7, 14, and 21 days). Thus, accelerated angiogenesis and its timely reduction are the therapeutic effects of wound healing materials.

Shen et al. examined the use of polysaccharides in the wound-healing process. The results demonstrated a significant change in the wound-healing process in the first week compared to a healthy group. Based on these results, polysaccharides extracted from *G. lucidum* can strengthen fibroblasts and change the C-terminal peptide of type I collagen and β1 growth factor in fibroblasts, resulting in wound recovery [39]. Similarly, our study demonstrated a significant difference in the wound recovery rate in the Al/col/Ps treatment group compared to positive and negative control groups. Based on this investigation, wound healing can be accelerated by increasing the number of fibroblasts and local growth factors.

Diniz et al. claimed that an alginate/gelatin-based hydrogel in combination with silver nanoparticles could significantly improve epidermis and collagen resynthesis rates, which effectively accelerated and contributed to wound healing compared to the control group [40]. Our microscopic examination also indicated significantly elevated collagen and epidermis, particularly on days 14 and 21, in comparison to the control group, which is similar to the study concerning the effect of the collagen/alginate hydrogel on the wound.

Zhang et al. presented evidence that the collagen/alginate hydrogel improved the extracellular matrix, adhesion structure, and cell proliferation, and maximized collagen synthesis. Collagen synthesis evaluation in different groups indicated the lowest synthesis rate in the positive control group [37]. The present study recorded the highest collagen synthesis and growth rate on days 14 and 21. The Al/col/Ps group's growth rate significantly differed from the positive and negative control groups ($P \leq 0.05$).

Shen et al. investigated the inflammation process in the initial days of wound healing. They found that the wound size decreased by reduced inflammation and wound healing progression [39]. Zheng et al. reported the anti-inflammatory role of polysaccharides, which mitigated the effect of inflammation by inhibiting the NLRP3 signaling pathway, thereby facilitating wound healing [41]. Consistent with previous research, the present results suggest the anti-inflammatory effects of drug compounds on wound healing. The inflammation rate decreased prominently on day 21, and the lowest rate was observed in the Al/col/Ps group. By inhibiting the NLRP3 signaling pathway and activating the Wnt/β-catenin signaling pathway, these compounds cause an earlier inflammation stage and termination of the wound healing process and, instead, the earlier onset of the proliferation phase of the wound healing process.

Conclusion

According to our results of the epidermis examination, the epidermis level increased gradually up to day 21. However, significant differences in the epidermis size were observed between the groups only on days 7 and 14. Compared to the control, polysaccharides accelerate wound healing by accelerating the granulated tissue formation. A greater depth of the granulated tissue indicates the faster shift of the wound to the proliferative

phase.

According to the present results and the comparison of treatment samples with positive and negative groups, it can be concluded that the local use of the Al/col/Ps hydrogel could accelerate the wound healing process, which played a significant role in wound closure, increased collagen levels, angiogenesis, fibroblastosis, hair follicle formation, epidermis, and inflammation reduction in the experimental wound of rats ($P \leq 0.05$). This study also demonstrated that the Al/col/Ps hydrogel was more effective than sulfadiazine in accelerating the shift from the inflammatory phase to the fibroblast hyperplasia phase due to elevated collagen synthesis and angiogenesis. Therefore, this mushroom can be used as a drug to help accelerate wound healing owing to its good efficiency, acceptable price, and availability.

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Conflict of Interest Disclosures

The authors have no conflict of interests to declare.

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Authors' Contributions

All authors contributed equally in the study.

Ethical Statement

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