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Original Article

Biological activities and wound healing potential of a water-soluble polysaccharide isolated from *Glycyrrhiza glabra* in Wistar rat

Atividades biológicas e potencial de cicatrização de feridas de um polissacarídeo solúvel em água isolado de *Glycyrrhiza glabra* em rato Wistar

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Abstract

The present study aimed to evaluate the *in vitro* antibacterial and antioxidant activities and the *in vivo* wound healing performance of a polysaccharide isolated from *Glycyrrhiza glabra* named PSG. It was structurally characterized by Fourier transformed infrared (FT-IR) spectroscopy, which confirmed the presence of different polysaccharides functional bands. The antioxidant capacity of PSG was determined *in vitro* and evaluated *in vivo* through the examination of wound healing capacity. Thirty two rats were randomly divided into four groups: group I was treated with physiological serum (negative control); group II was treated with "CYTOL CENTELLA®"; group III was treated with glycerol and group IV was treated with polysaccharide. The response to treatments was assessed by macroscopic, histologic, and biochemical parameters. Data revealed that our sample exhibited potential antioxidant activities and accelerated significantly the wound healing process, after ten days of treatment, proved by the higher wound appearance scores and a higher content of collagen confirmed by histological examination, when compared with control and "CYTOL CENTELLA®". Overall, these findings proved that this polysaccharide isolated from *Glycyrrhiza glabra* could be considered as a natural bioactive polymer for therapeutic process in wound healing applications.

Keywords: polysaccharide film, wound-healing, antioxidant activities.

Resumo

O presente estudo teve como objetivo avaliar as atividades antibacteriana e antioxidante in vitro e o desempenho cicatricial in vivo de um polissacarídeo isolado de *Glycyrrhiza glabra* denominado PSG. Foi caracterizado estruturalmente por espectroscopia no infravermelho com transformada de Fourier (FT-IR), que confirmou a presença de diferentes bandas funcionais de polissacarídeos. A capacidade antioxidante da PSG foi determinada in vitro e avaliada in vivo através do exame da capacidade de cicatrização de feridas. Trinta e dois ratos foram divididos aleatoriamente em quatro grupos: o grupo I foi tratado com soro fisiológico (controle negativo); o grupo II foi tratado com "CYTOL CENTELLA®"; o grupo III foi tratado com glicerol e o grupo IV foi tratado com polissacarídeo. A resposta aos tratamentos foi avaliada por parâmetros macroscópicos, histológicos e bioquímicos. Os dados revelaram que nossa amostra apresentou atividades antioxidantes potenciais e acelerou significativamente o processo de cicatrização da ferida, após dez dias de tratamento, comprovado pelos maiores escores de aparência da ferida e maior teor de colágeno confirmado pelo exame histológico, quando comparado ao controle e "CYTOL CENTELLA® ". No geral, esses achados provaram que esse polissacarídeo isolado de *Glycyrrhiza glabra* pode ser considerado um polímero bioativo natural para processos terapêuticos em aplicações de cicatrização de feridas.

Palavras-chave: filme polissacarídeo, cicatrização de feridas, atividades antioxidantes.

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1. Introduction

One of the most important worries in much pathology is the cutaneous wound healing. Also it is currently considered, as one of the recurring problems in skin damage (Hamdi et al., 2020). The wound healing is considered as a complex biological mechanism, which combined a series of events including hemostasis, followed by inflammation, proliferation, and remodeling (Miao et al., 2020; Pansara et al., 2020; Martinez et al., 1997). During these steps, inflammatory cells produce high amounts of reactive oxygen species (ROS), which are essential to protect the body against the development of infections. Therefore, several, studies were carried to find new natural polymers with potential antioxidant activity, which would accelerate and improve wound healing activity in the repairing process (Trabelsi et al., 2017; Chiarello, 1995).

In this context, polysaccharides have been widely used wound healing treatment due to their biocompatibility, low toxicity, and biodegradable characteristics compared with synthetic polymers (Luo et al., 2010). *Glycyrrhiza glabra* is an herbal plant that is composed with flavonoids, coumarin, and alkaloids, as well as poly glycyrrhizin, amino acids, sitosterol and polysaccharide. The latter is commonly used for the treatment of liver diseases due to its anti-inflammatory and protective effects (Samareh Fekri et al., 2021). However, several researches proved that Glycyrrhiza polysaccharides exhibit immunity regulation, phagocytosis, antivirus, antioxidants, antitumor, and low cellular toxicity activities (Zhang et al., 2015).

The present study focused on the investigation of antioxidant activities *in vitro* and the wound healing capacity of a novel polysaccharide extracted from *Glycyrrhiza glabra* named PSG. Wound healing efficiency *in vivo* of PSG was evaluated and it was examined by using a colorimetric assessment, collagen density and histological evaluation.

2. Materials and Methods

2.1. Materials and reagents

Glycyrrhiza glabra was sampled from Iraq/Baghdad in autumn (September/November) generally collected in the morning, and then identified by botanist. The leaves were carefully detached from the fresh plants, washed and dried in the shadow for 7 days at temperature room, then crushed to obtain a fine powder and stored in limp sterile.

2.2. Extraction of PSG

PSG was extracted using the method described by Liu et al. (2015). Dry vegetal material was pre-extracted with 95% ethanol to eliminate pigments. The dry residue was extracted twice with distilled water at 90 °C with continuous stirring for 4 h. Extracts were combined, filtered and evaporated under a vacuum. The concentrated liquid was precipitated with 95% (v/v) ethanol at 4 °C for 24 h and then centrifuged using a refrigerated centrifuge. The final precipitate was re-dissolved in double distilled water and freeze-dried using a freeze dryer (Bioblock Scientific Christ ALPHA 1–2, IllKrich-Cedex, France) to obtain polysaccharide. The yield was expressed as percentage (%) of the mass (g) of PSG against the initial mass (g) of plant powder.

2.3. Spectroscopic analysis of PSG

2.3.1. FT-IR spectrometric analysis

IR spectroscopy of PSG was carried out into a Nicolet Nexus spectrometer. Spectrum was obtained at a resolution of 4 cm^{-1} and the measurement range was 4000–500 cm^{-1} at room temperature. The spectral data were analyzed by the OPUS 3.0 data collection software (Bruker, Ettlingen, Germany).

2.3.2. Physical analysis

Color, pH, and viscosity of PSG were determined. The color was evaluated using a Color Flex spectrocolorimeter (Hunter Associates Laboratory Inc., Reston, VA, USA) and reported as L*, a* and b* values, referring to the parameters of lightness, redness, and yellowness, respectively. pH (solution of 1%) was measured using a digital pH meter with complete immerging of the glass electrode into the solution. Viscosity measurements were determined at 25°C by using a digital viscometer (NDJ-1, Japon) at 30 rpm spindle rotation.

2.4. In vitro antioxidant properties of PSG

2.4.1. DPPH free radical scavenging assay

The DPPH radical scavenging activity was determined following Lopes-Lutz et al. (2008). The absorbance was measured at 517 nm with a spectrometer. The percent of inhibition (PI) was calculated using the Equation 1 below:

$$\mathrm{PI}(\%) = \frac{\mathrm{Ac} + \mathrm{Ab} - \mathrm{As}}{\mathrm{Ac}} \times 100 \quad (1)$$

Where, Ab, Ac and As refer to the blank, control and sample optical densities respectively. All experiments were performed in triplicate with Gallic acid as positive control.

2.4.2. Ferrous iron chelating activity

The chelating activity of PSG toward ferrous ions (Fe^{2+}) was determined according to Decker and Welch (1990). The decrease in the red color of (Fe^{2+}-ferrozine) complex was measured at 562 nm. The percent of ferrozine-Fe^{2+} complex formation was calculated as follows (Equation 2):

$$\text{Ferrous ion chelating activity}(\%) = \frac{(\text{A control} + \text{A blank} - \text{A sample})}{\text{A control}} \times 100 \quad (2)$$

2.4.3. Total antioxidant activity

The total antioxidant activity of PSG was determined according to Prieto et al. (1999). The absorbance was measured at 695 nm against a blank.

2.5. In vivo experimental study

2.5.1. Animals

Thirty two Males adult Wistar rats were used in this protocol, weighing between 150 and 200 g. All rats were

maintained at normal room temperature (22–24 °C) on a 12 hours light/dark cycle, with free access to food and water. The animals were kept in individual cages. All animals' procedures were conducted in accordance with the Guide for the Care and Use of Laboratory of Animals issued by the University of Sfax, Tunisia, and approved by the Committee of Animal Ethics (Council of European Communities, 1986).

2.5.2. Excision wound model

The excision model was used for the evaluation of wound contraction in this protocol. Animals in each group were anesthetized by hydrate chloral. The rats were depilated on the back. The circular wound, approximately 1 cm × 1 cm (150-200 mm^2), was created on the dorsal region of each animal by excising the skin.

2.5.3. Experimental design

After the excision of skin, 32 animals were divided into four groups of eight animals each:

- Group 1: rats were treated with a saline solution (0.9%), and used as a control group;
- Group 2: rats were treated with a standard drug "CYTOL CENTELLA" cream;
- Group 3: rats were treated with glycerol;
- Group 4: rats were treated with glycerol+PSG.

The treatments were applied every two days till the wounds were completely healed. On day 10, all the rats were anaesthetized with ether, euthanized by decapitation and the granulation tissues were excised from the euthanized animals.

2.5.4. Measurement of wound area and contraction rate

During the treatment period, all wounds were examined by digital photography, and the wound area was traced manually. The wound surface areas were measured using Autodesk Auto CAD for designing and drafting. The wound contraction rate was calculated according to the following Equation 3:

$$\text{Rate of wound contraction } (\%) = \frac{\text{intial surface size} - \text{specific day surface size}}{\text{intial surface size}} \times 100 \quad (3)$$

2.5.5. Determination of hydroxyproline content

The measurement of Hydroxyproline content was determined according to Edwards & O'Brien Junior (1980). The absorbance was measured at 557 nm. The results were reported as mg/g dry weight of tissue.

2.5.6. Histological examination

After the euthanasia of rats, biopsies from the wound site of all animals'- groups were obtained and fixed immediately in neutral-buffered formalin solution (10%), embedded in paraffin wax, cut into 5 µm thick sections and colored with hematoxylin-eosin then examined and photographed under a light microscope (Olympus CX41, Tokyo, Japan).

2.6. Statistical analysis

Statistical analyses were performed with Graph Pad Prism version 9.0, it is a professional edition using ANOVA analysis at a p level=0.05. A standard deviation at the 95% confidence level was used to compare all parameters.

3. Results and Discussion

3.1. Spectroscopic analysis of PSG

3.1.2. FT-IR spectrometric analysis

FT-IR spectrometric analysis was used to determine functional groups of PSG absorbance from 4000 to 400 cm^{-1}. As shown in Figure 1, the FT-IR spectrum revealed a large peak at 3285.21 cm^{-1} and a weak peak at 2935.03 cm^{-1} which referred to the stretching vibration of O-H and C-H groups, respectively (Jaballi et al., 2019). The two peaks at 1416.97 and 1633.19 cm^{-1} could be assigned to C=O stretching vibration of carboxylic groups (Huang et al., 2020). The absorption band at 1020.49 cm^{-1}could be assigned to the vibration of the (COH) groups and the (COC) glycosidic bond vibration (Ktari et al., 2017a). Finally, the small peak at 567 cm^{-1} reflected the pyranose structure of PSG (Baranov et al., 2021).

3.2. Physical analysis

Physical properties of PSG such as pH, color, and viscosity are presented in Table 1. The color parameters of PSG were determined such as L* (lightness), a* (redness) b* (yellowness). However, our sample characterized by a light (L* = 90.76), a red color (a* = 0.5±0.01) and a yellow color (b* = 5±0.05). Moreover, 1% of PSG solution pH at 25 ◦C was about 7.1 ± 0.1. Whereas, the viscosity increased with the increase of concentration.

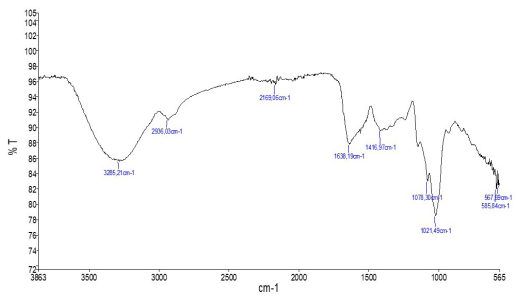

Figure 1. FT-IR spectra of PSG.

Table 1. Physical properties of polysaccharide PSG.

Color	PSG
L*	71.2± 0.05
a*	0.5± 0.01
b*	5± 0.05
0.5 g/l	4.6± 0.05
1 g/l	6.5± 0.45
1.5 g/l	8.5± 0,2
pH (1% solution at 25°C)	7.1± 0.1

3.3. In vitro antioxidant properties of PSG

3.3.1. DPPH free radical scavenging assay

DPPH is the most important free radical usually used to evaluate the free-radical scavenging capacity. Our results exhibited a significant antioxidant ability of PSG which increase with the increase of polysaccharide concentration. Our polysaccharide revealed a maximum DPPH inhibition (64% ±0.05) (Figure 2A) at a concentration of 2.5 mg/ml of PSG. In this context, several studies revealed that the antioxidant capacity of polysaccharides, in general, is highly related to their monosaccharide compositions, molecular weight, and configuration (Hamzaoui et al., 2020; Feki et al., 2020). In the same way, the radical scavenging capacity of our sample could be due to the presence of functional groups such as hydroxyls and carboxyls in polysaccharide composition, which played the role of hydrogen donator for scavenging the DPPH free radical, thus reducing the effect of oxidative stress (Feng and Zhang, 2020).

3.3.2. Ferrous iron chelating activity

Metal chelating activity is indicated as one of the antioxidant mechanisms, which reduced the level of the catalyzing transition metal in lipid peroxidation (Qiao et al., 2009). In this study, the metal chelating capacity was determined using EDTA as a standard positive. Figure 2B revealed that PSG has an interesting activity, which increased with the increase of concentrations. The maximum activity around 74% ± 0.02, was observed at a concentration of 10 mg/ml of polysaccharide. The presence of reducers as antioxidants indicated the transformation of the Fe^{3+}/ferric cyanide complex to the ferrous form. In this context, many previous studies confirmed the correlation between antioxidant activities and reducing power assay (Qiao et al., 2009).

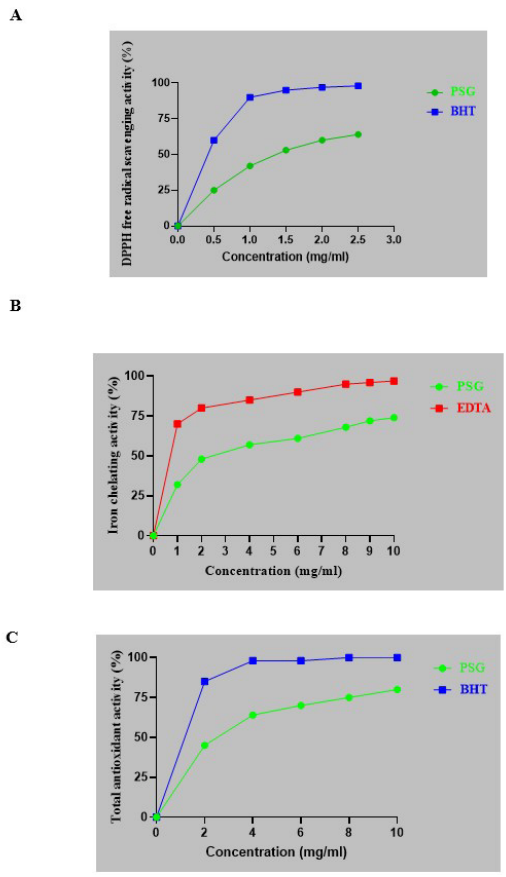

Figure 2. DPPH free radical scavenging assay (A), ferrous iron chelating activity (B), and total antioxidant activity of PSG (C).

3.3.3. Total antioxidant activity

Our results showed a potent total antioxidant capacity of PSG, increasing proportionally with the augmentation of the concentration as shown in Figure 2C. Our sample revealed a maximum activity (80% ± 0.05) at a concentration of 10 mg/ml of polysaccharide.

3.4. In vivo experimental study

3.4.1. Morphological evaluation

Wound healing activities were followed after the circular excision wound model and it was daily observed for ten days. Figure 3 illustrated the representative photographs captured on days 1, 3, 5, 7, and 10. On the wound induction day, bright red color reflecting the blood that covers the underlying muscle was observed in all wounds. From the third day of treatment, the untreated wounds revealed a higher inflammatory side around the injured skin, though a brown color showed in the treated groups (PSG and "CYTOL CENTELLA"). This color is related to scab formation, which is declared the initiation of the healing process by the development of blood clots with a remarkable reduction in the area of wounds. On the 7th day of treatment, the area of wounds decreased and showed a pinkish-colored tissue for the groups treated with polysaccharide and the standard drug. Additionally, an accentuated inflammation is still detected in untreated rats (physiological serum and glycerol) with dark red color. On the 10th day, a complete closure of the wounds was observed in the group treated with PSG. Although, the open wound and scabs are stilled observed in the untreated groups. These results underlined the healing capacity of PSG and attested to the potential role of polysaccharide in acceleration wound healing process. These data are in accordance with those obtained by Ktari et al., 2017b.

3.4.2. Assessment of wound closure

The wound contraction was evaluated by measuring daily the size of the areas wound during the experimental period. As shown in Figure 4, the contraction rates of the wounds treated with PSG and "CYTOL CENTELLA" were better than that of the control groups (treated with physiological serum and glycerol). Rats treated with the polysaccharide and the standard drug revealed an important healing potential, beginning on the 3^{rh} day compared with the other interested groups. On the 10th day of treatment, the wound of the untreated groups was still open (96.23± 0.25% and 95.13± 0.095%) for the groups treated with physiological serum and glycerol, respectively,

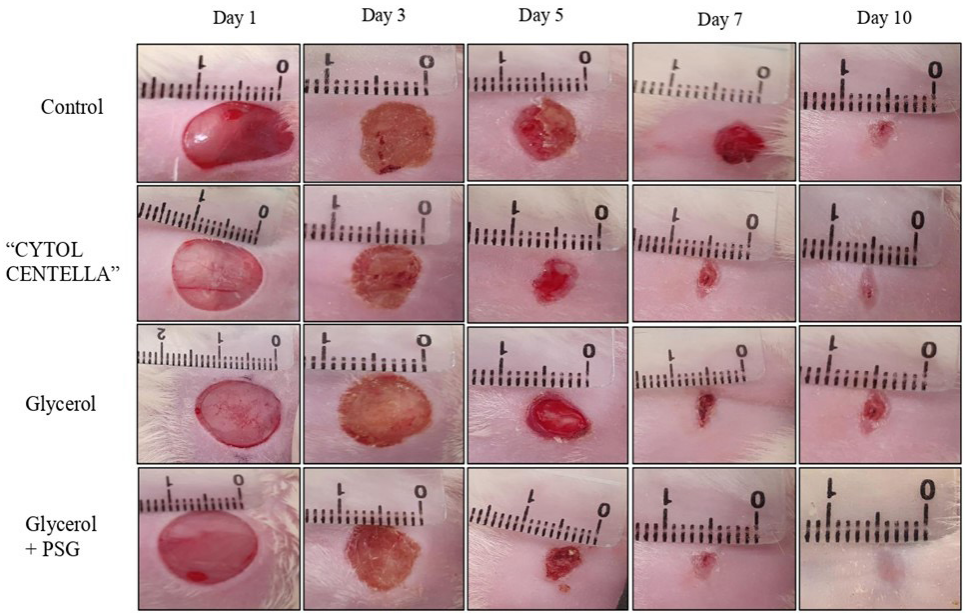

Figure 3. Representative photographs of macroscopic appearance of 1 cm × 1 cm wounds excised on the rats on days 1, 3, 5, 7, and 10 of group treated with physiological serum, "CYTOL CENTELLA", glycerol and PSG + Glycerol.

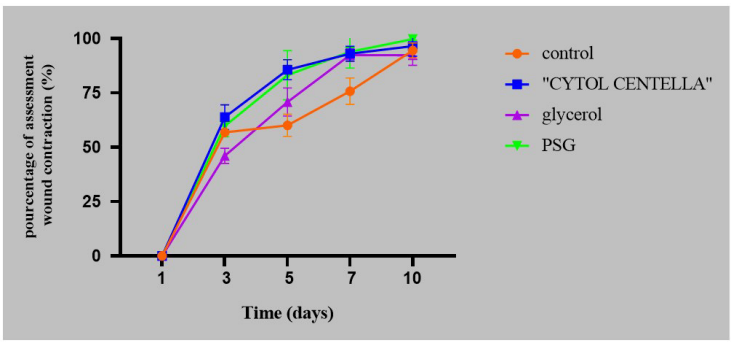

Figure 4. Percentage of wound areas contraction of different group of rats treated with physiological serum, "CYTOL CENTELLA", glycerol and PSG + Glycerol on days 1, 3, 5, 7, and 10.

which healed tardily compared with the other groups. Meanwhile, the rats treated with the polysaccharide showed a total size reduction in the wound site (100%) compared with control and total re-establishment of the initial skin. These data proved that our sample accelerates wound reparation as a major step in the wound healing process. The accelerated wound contraction obtained by the polysaccharide could be due to its ability to stimulate inflammatory cells and fibroblasts generation in wound location (Moyer et al., 2002).

3.4.3. Estimation of hydroxylproline content

Collagen, the major structural protein of extracellular tissue in the body, is composed of hydroxyproline that was used as a biochemical marker for collagen tissue (Trabelsi et al., 2017; Greca et al., 1997). Indeed, the assessment of alteration of collagen reflects the process of wound healing in the damaged tissue (Ktari et al., 2017b). As shown in Table 2, higher levels of hydroxyproline were detected in the groups of rats treated with PSG (1315.75± 10.87) and "CYTOL CENTELLA" (1165.08± 5.43) ($p<0.05$), when compared with the control (489.08± 16.31) and glycerol treated group (531.37± 32.62). These data suggested that polysaccharide is able to stimulate the wound healing process by activating fibroblast and collagen synthesis in the wound site. In this context, Trabelsi et al. (2017) and Ktari et al. (2017b) have reported that the high concentration of hydroxyproline can be due to the capacity of the polysaccharide to promote fibroblast migration by providing a connective tissue matrix.

3.4.4. Histological evaluation

The histological observations of wound tissues are one of the methods used to evaluate the degree of wound healing as shown in Figure 5. The micrograph sections are colored with hematoxylin and were photographed

Table 2. Hydroxyproline amount in biopsies from experimental rat wounds.

Groups	Hydroxyproline amounts (mg/g of tissue)
Control	489.084± 16.31[a]
Glycerol	531.379± 32.62[a]
CYTOL CENTELLA	1165.085± 5.43[b]
GLYCEROL+ PSG	1315.75± 10.87[c]

Results are expressed as mean of three experiments ± SD. The number of determinations was n =3. a, b and c in the same column indicate significant differences ($p < 0.05$).

by light microscopy for the assessment of inflammatory and regenerative properties such as tissue congestion, neutrophil infiltration, granulation tissue formation, and neovascularization (Suh et al., 2016). The histological evaluation of the epidermis revealed an incompletely re-epithelization in the untreated group and the group treated with glycerol. Furthermore, the dermis showed an invasive inflammatory infiltrate with necrosis and a low level of collagen and fibroblast formations. Nevertheless, the histological observations of the wounds treated with PSG and "CYTOL CENTELLA" revealed a better wound re-epithelization, and neovascularization with the presence of few macrophages, affirming the supplying of connective

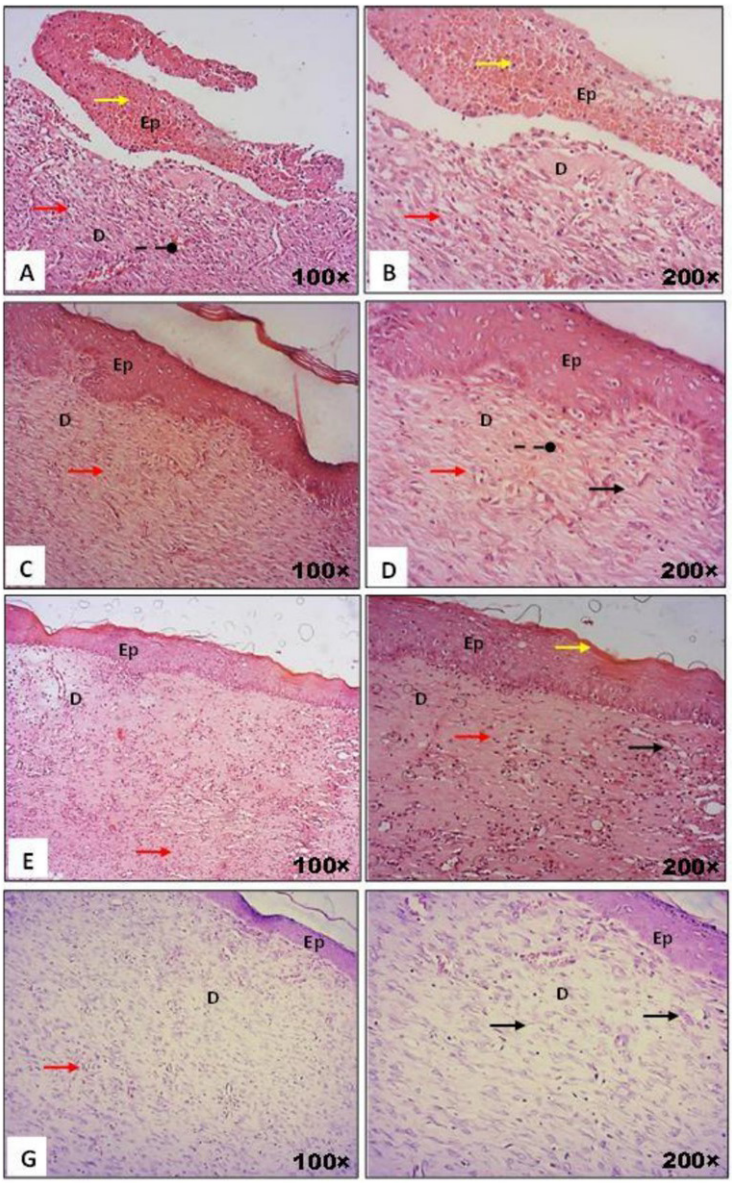

Figure 5. Histological changes of the dermal tissue of Wistar rats stained with H&E at magnification (x100) and (x200) in physiological serum **(A-B)**, "CYTOL CENTELLA" **(C-D)**, glycerol **(E-F)** and PSG + Glycerol **(G-H)**. **Ep:** Epidermis; **D:** Dermis; ⟶ : Collagen formation; ⟶ : Ulceration; – –● : Hemorrhage; ⟶: Inflammatory infiltrate.

Table 3. Histopathological scores on healed wound sections isolated from rats from each group.

Groups	Inflammatory infiltrate	Fibroblast proliferation	Collagen formation	New vessels	Epithelium	Epidermal differentiation	Total score
Physiological serum	2	2	1	2	3	3	13
Glycerine cream	2	3	1	2	2	3	13
Cytol centella	2	2	2	3	3	4	16
Glycerine cream + PSG	3	3	3	3	4	4	20

tissue associated with the interesting elevation and a better organization of collagen and fibroblast fibers. This finding indicated a final step of remodeling phase healing and the healing process was accomplished with an excellent dense structure. These results suggested that our sample generates an advantageous effect on cutaneous wound repair. However, a significant relationship between monosaccharide structure in PSG and wound healing capacity, which could induces a pro-inflammatory effect that may stimulate the wound healing mechanism, was noted (Ghlissi et al., 2020). These data are confirmed by histopathological scores calculated for each group sections (Table 3). Our result revealed that the group treated with the PSG displayed the highest score for epithelialization and epidermal differentiation. This fact indicated a final step of remodeling phase healing. Additionally, previous reports demonstrated that Free radicals and oxidative reaction products in lipid peroxidation induce tissue destruction and play a major role in the irritation of tissue (Eleroui et al., 2021). In this context and in accordance with above obtained *in vitro* antioxidant activities, it may be concluded that our sample is an antioxidant agent, which can quenches free radicals by electron-donating mechanism, supplying a protective effect of cells from oxidative damage in wound healing process (Eleroui et al., 2021; Carmignan et al., 2019).

4. Conclusion

This study reports the wound healing capacity and the antioxidant activity of PSG extracted from *Glycyrrhiza glabra*. According to our data, our sample accelerated the re-epithelialization and wound closure in the excisional wound model in Wistar rats. PSG was also found to exhibit strong antioxidant activity in *vitro*. In general, these findings suggested that PSG could be a novel perspective for the therapeutic process in wound healing applications.

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