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Research Article

EFFECT OF PRODIGIOSIN FROM *SERRATIA MARCESCENS* BR1 STRAIN AS AN ANTIOXIDATIVE, ANTIMICROBIAL, AND *IN VIVO* WOUND HEALING

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ABSTRACT

Objective: The aim of this work is to evaluate antioxidative, antimicrobial, and healing wound potential of prodigiosin extracted from *Serratia marcescens* strain microbiota of a traditional Algerian fermented cereal food. The goal is to develop a natural galenic formulation for external use.

Methods: After extraction and purification of the red pigment, the Fourier transform infrared spectrum is determined. The antioxidative activity was performed by scavenging radical with 2-diphenyl-1-picrylhydrazyl (DPPH), bleaching of beta-carotene, and ferric reducing antioxidant power. Antimicrobial tests were assessed against bacteria and fungi pathogenic reference strains *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 10541, *Klebsiella oxytoca* ATCC 13182, *Staphylococcus aureus* CC 10541, *Helicobacter pylori*, and *Candida albicans* ATCC 10231. Healing wound activity was achieved *in vivo* on Wistar rats using as a reference to the commercial formulation Madécasol.

Results: *S. marcescens* BR1 produce a prodigiosin where IR spectrum is typical. The DPPH test shows a trapping power of 80% at 1 mg/ml and an inhibitory concentration 50 equal to 0.54 mg/ml. The discoloration of β-carotene is 50% with high ferric reducing antioxidant power (FRAP). *Candida albicans* were the most sensitive to prodigiosin with inhibition diameters >20 mm. All strains tested are sensitive to prodigiosin. *C. albicans* were the most sensitive with inhibition diameters >20 mm followed by *H. pylori* strain (15 mm) and *E. coli* (13.5 mm). Prodigiosin ointment at 0.1% in Vaseline was used to achieve *in vivo* healing activity. Obtained results showed a fast and effective wound healing potential, better than the standard (Madécasol). The cicatrization traces totally without any of the lesions. We discovered the absence of the redness phase. This formulation, based on prodigiosin, is very promising as a natural replacement for the synthetic drug, having powerful anti-microbial, wound healing, and anti-inflammatory activities.

Conclusion: A based cream of prodigiosin has a rapid, powerful wound healing potential with a dose-dependent effect. It is very promising as a natural replacement for the synthetic drug.

Keywords: Antioxidative, Antimicrobial, Healing activity, Prodigiosin, *Serratia marcescens*.

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INTRODUCTION

Wound healing is a natural biological process whose speed and quality depend on many factors. The general condition of the affected organism, etiology, location, and infection occurs, as well as genetic factors predisposing or not to cicatrization disorders [1]. Sumathi *et al*. [2] report the severely hampered by microbial infection and reactive oxygen species (ROS). The treatment of wounds is still controversial restricted as simplification with a questioning of the systematic use of drug treatments. Despite the existence of a multitude of healing products whose effectiveness is established, the fact remains that many authors are testing the healing activity of new products, most often chosen from medicinal plants because of their effectiveness, their diminished side effects, and their relatively low cost [3]. Although there are a few numbers of them, available in sufficient quantities to be useful for industry because they are usually extracted from plants [4].

Moreover, microorganisms contain a huge potential of therapeutic substances. Among them, pigments produced by bacterial flora play a significant role in human health care. Bacterial pigment production is now one of the emerging fields of research to demonstrate its potential for various industrial applications [5]. Pigment production from microorganisms is easy and fast growth in the cheap culture medium, independence from the weather. Bacterial pigments are much more stable than those of plant origin [6].

Recently prodigiosins attracted much research due to clinical importance. Prodigiosins are a secondary metabolite of some bacteria such as *Serratia* sp., *Pseudomonas* sp., and *Vibrio* sp. *Serratia marcescens* strains are the major producers of prodigiosin [7,8]. The red color comes from a tripyrrolic structure with seven conjugated double bonds. It is part of a family of molecules called "prodigiosins" characterized by their pyrrolyl dipyrromethene unit and the presence of a methoxy (or hydroxyl) group. Structural variations are particular the nature and position of the alkyl chains [9]. The prodigiosin family has many biological activities; anti-cancer, immunosuppressive, antifungals, antibacterials, anti-protozoa, and antimalarials activities [10]. The present investigation includes the isolation of prodigiosin from *S. marcescens* BR1 strain, evaluation of antimicrobial, and antioxidant activity as part of wound healing activity.

METHODS

Isolation and identification of the strain

The strain was previously isolated from BEROUIL, an Algerian traditional cereal fermented food by the standard microbiological method and maintained at −80°C in LB broth (Luria and Bertani, Merck) containing 20% (v/v) glycerol (Sigma Aldrich). Working cultures were prepared in LB broth at 30°C for 18–24 h. The strain was identified by the API 20E gallery (Bio Mérieux) and matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) MS to confirm the identity (Bruker Daltonik MALDI Biotyper: Germany). Identification was conducted directly on the bacterial young colonies.

Extraction and purification of prodigiosin

The extraction of prodigiosin from bacteria was performed according to the protocol of Ramani *et al.* [11]. The pellet of 36 h old culture

on LB broth was suspended in an equal volume of ethanolic acid (95 ml ethanol 95% and 4 ml HCl1M) after vortexed, centrifuged at 5000 rpm for 15 min. The filtrated supernatant (Whatman 0.2 µm filter) was concentrated under vacuum in a rotary evaporator (Buchi Rotavapor R-300, Germany) at 45°C. The crude extract was purified by dissolving in 10 ml of chloroform and passed through silica gel column chromatography [12].

Dynamic growth and prodigiosin production

An overnight preculture on LB broth serves as an inoculum. The culture was carried out in 1 L Erlenmeyer flask filled with 1/10 of their volume by the sterile LB broth. Inoculated at a rate of 10% (v/v). The experiment was conducted under the same conditions as preculture (incubation at 30°C/ agitation rate 160 rpm/mn) in triplicate. Samples of 10 ml at each time interval were taken. The biomass was calculated using OD600 of broth versus dry cell weight standard curve. The absorbance of appropriately diluted purified prodigiosin samples was measured at 535 nm and the content was calculated using the prodigiosin standard curve [13].

Fourier transform infrared (FTIR) spectroscopy of purified prodigiosin

The FTIR spectroscopy spectrum of the prodigiosin was done with a spectrometer infrarouge FTIR – 8400 SHIMADZU in the range of 400–4000 cm^{-1}.

Evaluation of the antioxidant activity of prodigiosin

2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging test

The radical scavenging method using DPPH radical determined the antioxidant activity of the samples and standards (DPPH). About 0.1 ml of methanolic solutions of the samples or standards at different concentrations was each added to 3.9 ml of a DPPH methanolic solution (0.2 mM). These concentrations were selected due to the linear range of DPPH solutions.

Scavenging effect (%) = [1-(Abs control – Abs sample)/Abs control] × 100

Where Abs control is the absorbance of the control $DPPH_o$ solution without extracts and Abs sample is the absorbance of the sample. The blank sample consisted of 0.1 ml of methanol added to 3.9 ml of DPPH. The trials were held out in triplicate. After 90 min incubation period at room temperature in the dark, the absorbance was measured at 517 nm. Efficient inhibitory concentration 50 (IC_{50}) have been graphically calculated [14].

***β* carotene bleaching test**

This test is carried out by measuring the inhibition of volatile organic compounds and the diene-conjugated hydroperoxides resulting from the oxidation of linoleic acid [15]. Absorbance was immediately measured for BHT (control) at 490 nm. Tubes were placed in the dark at room temperature for 48 h. A second reading was conducted at 48 h. The relative antioxidant activity after 48 h was estimated according to the following relation:

RAA = Abs simple (48 h) Abs BHT (0 h) ×100

RAA: Relative antioxidant activity; Abs Sample (48 h): Absorbance of the sample after 48 h; Abs BHT (0 h): Absorbance of BHT at t = 0 h.

FRAP

The reducing power of iron (Fe3+) of purified prodigiosin was determined according to the method described by Oyaizu [16]. The results were expressed in IC_{50}, which defined as the concentration of antioxidants required to reduce 50% of the initial concentration of ferric thiocyanate. Optical density was performed at 700 nm.

Evaluation of antimicrobial susceptibility

The antimicrobial activity of prodigiosin was assessed by agar well diffusion assay. The tested bacteria were *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 10541, *Klebsiella oxytoca* ATCC 13182, *Staphylococcus aureus* CC 10541, *Klebsiella pneumoniae*, *Bacillus cereus*, *Streptococcus pyogenes*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, and *Helicobacter pylori* (collection of laboratory). All bacteria strains are antibiotic-resistant. Fungi strains tested were *C. albicans* ATCC 10231, *C. albicans* ATCC 26790, and *C. albicans* IP 444 (Institut Pasteur, France). Twenty microliters of filter-sterilized (0.22 µm Millipore) prodigiosin solution were inoculated in the wells of Mueller–Hinton soft agar containing an overnight culture of the target microorganisms. *C. albicans* strains were inoculated on PDA (Potatoes Dextrose Agar Difco). The plates were incubated at 37°C for 24 h. At the end of incubation, the inhibition zones were recorded.

***In vivo* evaluation of wound healing activity**

All experimental procedures involved in this research were according to the approved protocols by the Animal Ethics Committee of Hassiba Benbouali University. Incision and excision wounds were inflicted on albino rats (180–200 g) of both sex previously locally anesthetized and dehaired. Incision wounds of about 2 cm^2 in area and 0.5 cm in depth were created with a scalpel blade and cleaned with sterile cotton before application of the ointment. Xylocaine 2% was used as an anesthetic at the concentration of 3 mg/kg (calculated according to the weight of the animals). Animals were divided into four groups, each containing six animals: The first group was shaved and disinfected with ethanol without incision, the second was incised and disinfected, the third was incised and treated with Madécasol (0.1 mg), and the fourth was incised and treated with prodigiosin (0.1% prodigiosin in Vaseline). The wound dressing was renewed daily for the 1st week, and after every 3rd day for the 2nd week. Once a day with a precise amount of ointment (about 0.50 g) applied to wounds cleaned with 70% ethanol. Wound measurements were conducted every 3 days until complete wound healing. The appearance and color of the wounds were noted throughout the duration of the treatment. Percentage narrowing (Pn) of wounds was determined according to the following formula:

$$Pn = \frac{Md1\text{-}Mdn}{Md1} \times 100$$

MD1: Measure 1st day, Dn: Measure n^{ieme} day.

Statistical analysis

All experiments were done in triplicate. Standard deviations were calculated and included in the graphical representation of the data. The data were analyzed by an analysis of variance ($p<0.05$). The IC_{50} values were calculated from a linear regression analysis.

RESULTS

Isolation and identification of the strain

Bernoulli isolate showed a Gram-negative, rod-shaped bacterium. Results of the biochemical identification tests (API 20 E) matched best with the genus Serratia and showed 99% similarity to *S. marcescens*. A log score >2.30 was obtained with MALDI-TOF MS, which indicated highly probable species identification as *S. marcescens* strain.

Representative MALDI-TOF MS spectra for *S. marcescens* strain (Fig. 1), the M/z values for prominent peaks were displayed.

Dynamic of growth and prodigiosin production

As presented in Fig. 2, results suggested that the production of prodigiosin from Serratia marcescens BR1 strain in LB broth was growth dependent.

An increase in pH of the medium is observed from 7, 5 at the beginning of the cultivation to 9, 8 after 72 h of incubation. Revealing that to produce significantly intracellular red pigment, bacterial cells need alkaline conditions (Fig. 3).

The FTIR spectrum for the prodigiosin shows broadband located in the range of 3700–3000 cm^{-1}, with intense and very characteristic peaks

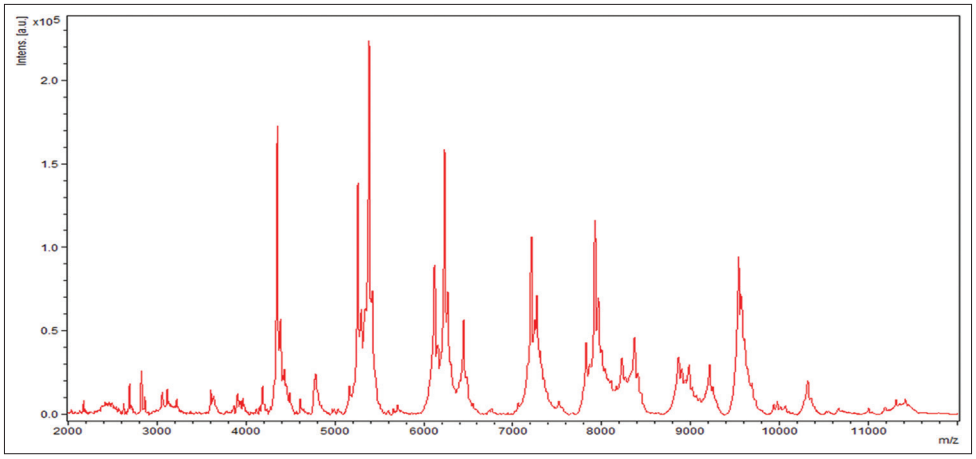

Fig. 1: MALDI-TOF MS spectrum for *Serratia marcescens* BR1 strain

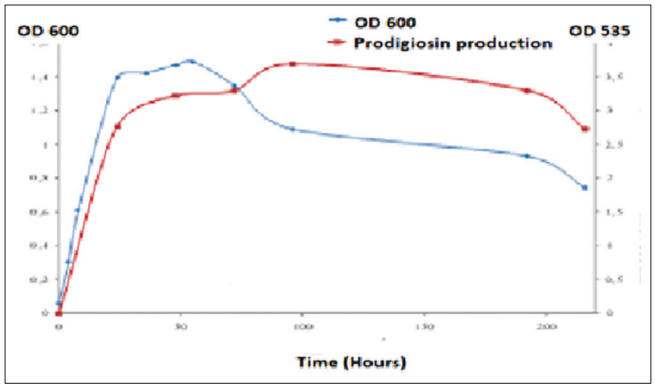

Fig. 2: Growth and prodigiosin production by *Serratia marcescens* BR1 in LB broth at 30°C agitated rate of 160 rpm

at 3335,28. Latter peaks correspond to the valence vibrations of the O-H and N-H (Amide II) functions of the prodigiosin. The secondary amides are linked by H bond to form dimers (CIS configuration) or polymers (trans-configuration) result of the replacement of the N-H band.

Antioxidative activity of prodigiosin

The DPPH radical scavenging capacity of ethanolic extract prodigiosin is done in comparison with ascorbic acid. Ascorbic acid showed high activity with IC_{50} from a concentration of 0.25 mg/mL. The radical scavenging activity of prodigiosin on DPPH showed high activity with dose-dependent and is found to be 80% at the concentration of 1 mg/ml and has IC_{50}<0.60 mg/ml (Figs. 4 and 5).

The total antioxidant activity can be measured by the FRAP assay. Higher FRAP values give higher antioxidant capacity. The obtained result shows prodigiosin as a good electron donor and could terminate the radical chain reaction by converting free radicals to more stable products.

The β-carotene decolonization power of prodigiosin obtain is 38.66% at 4 mg/ml, the same as BHT. Over this concentration. BHT shows more efficiency with a maximum level of inhibition of 91.75% at 1 mg/ml (Fig. 6).

Antimicrobial activity

As indicated in Table 1, both bacteria (Gram-positive and Gram-negative) and fungi were sensitive to prodigiosin. Inhibition zone observed was high with *C. albicans* strains followed by *H. pylori*, *E. coli*. *S. aureus*, and *P. aeruginosa* at least.

Table 1: Antimicrobial activity of the prodigiosin extracts from *Serratia marcescens* BR1

Target strain	Inhibition zone (mm)		MIC µg/ml
	Control	Extract	
Staphylococcus aureus (ATCC 10541)	00	9±0.33	0.51
Escherichia coli (ATCC 25922)	00	13.5±2.12	0.12
Enterococcus faecalis (ATCC 10541)	00	11±0.22	0.35
klebsiella oxytoca ATCC 13182	00	7±1.25	0.88
klebsiella pneumoniae	00	10.25±0.35	0.70
Bacillus cereus	00	11.5±0.7	0.66
Streptococcus pyogenes	00	12±0.75	0.56
Helicobacter pylori	00	15±1.41	0.45
Proteus mirabilis	00	12.5±1.41	0.45
Pseudomonas aeruginosa	00	10.75±2.47	0.87
Candida albicans IP 444	00	24.5±0.70	0.41
Candida albicans ATCC 10231	00	23.75±1.76	0.42
Candida albicans ATCC 26790	00	20±0.5	0.45

***In vivo* wound healing assays**

Obtained results (Figs. 7 and 8) showed a fast and effective wound healing potential, better than the standard (Madécasol).

DISCUSSION

In the present work, the authors report the wound healing potential in the excised wound model on Wistar rats of prodigiosin extracted from *S. marcescens* BR1 strain. The isolate of Broil, a popular Algerian traditional fermented cereal food, is a speed growth – producer of the red pigment appearing earlier in the stage of bacterial growth. In cultivation conditions (LB broth/30°C/160 RPM), lag phase not exist. These results suggested that the red pigment production is growth dependent. Darah *et al.* [17] noticed the same phenomena as marine *S. marcescens* IBRLUSM 84. The purified red pigment evaluation revealed antimicrobial activity on various pathogenic strains, as reported in several studies. *In vitro* analysis showed a potential inhibitory effect against Gram-positive bacteria, Gram-negative bacteria, and a strong effect on fungi. This may be attributed to the fact that these two groups differ in their structure of the cell wall components. Inflammatory processes are provoked by free radicals as important mediators. Antioxidants are used for neutralizing them to prevent and treatment in case of complex diseases such as atherosclerosis, stroke, diabetes, Alzheimer's disease, and cancer [18]. Recently, interest has increased considerably in finding naturally occurring antioxidants to replace synthetic antioxidants, which are being restricted due to their carcinogenicity [19]. In this investigation, the antioxidant potential of

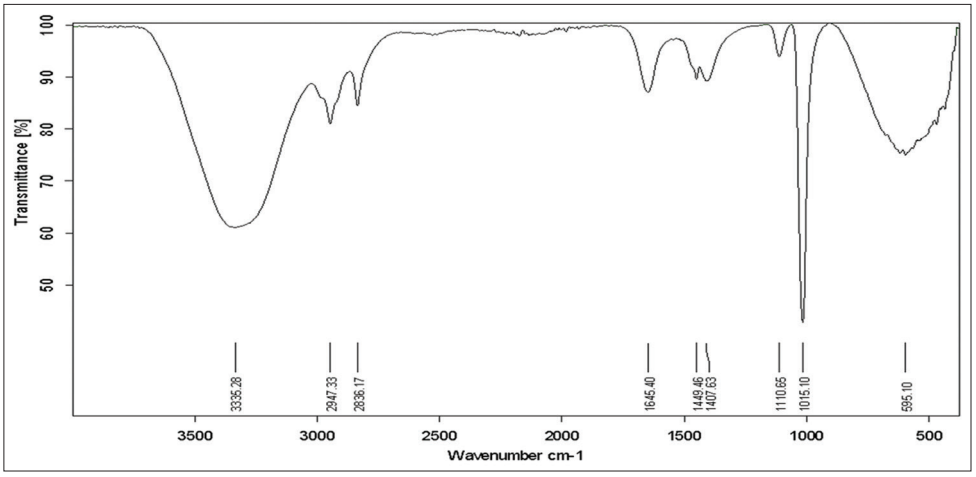

Fig. 3: FTIR spectral of prodigiosin from *Serratia marcescens* BR1 strain

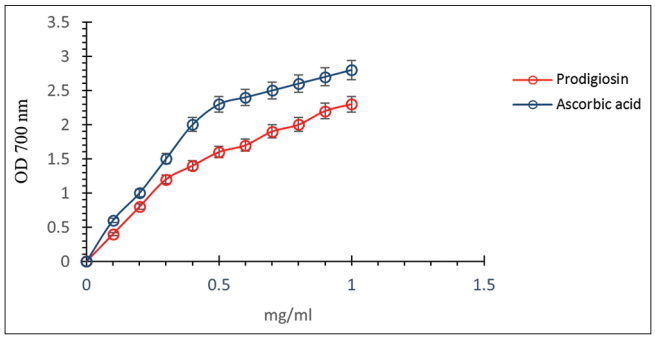

Fig. 4: Scavenging effect of prodigiosin on 1,1 diphenyl-2-picrylhydrazyl (DPPH) radicals. Each value represents the mean ± standard deviation (n=3).

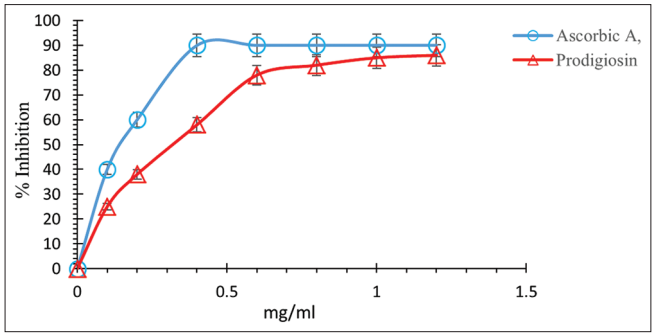

Fig. 5: Ferrous ion chelating effect of prodigiosin. Each value represents the mean ± standard deviation (n=3).

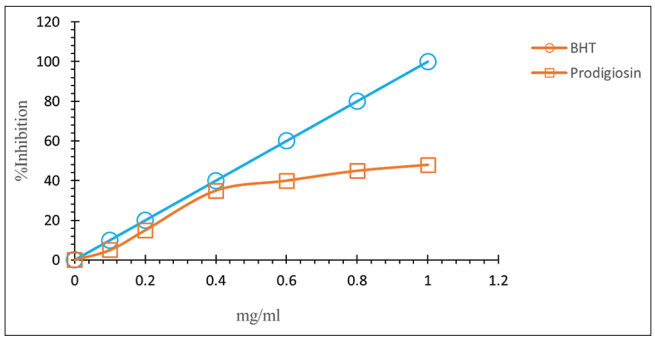

Fig. 6: *β* carotene blanching power of Prodigiosin compared to BHT. Each value represents the mean ± standard deviation (n=3).

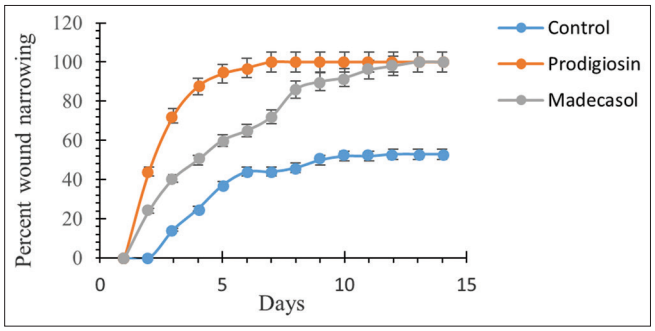

Fig. 7: Wound-narrowing values of wound healing on Wistar rats. Each value represents the mean ± standard deviation (n=3).

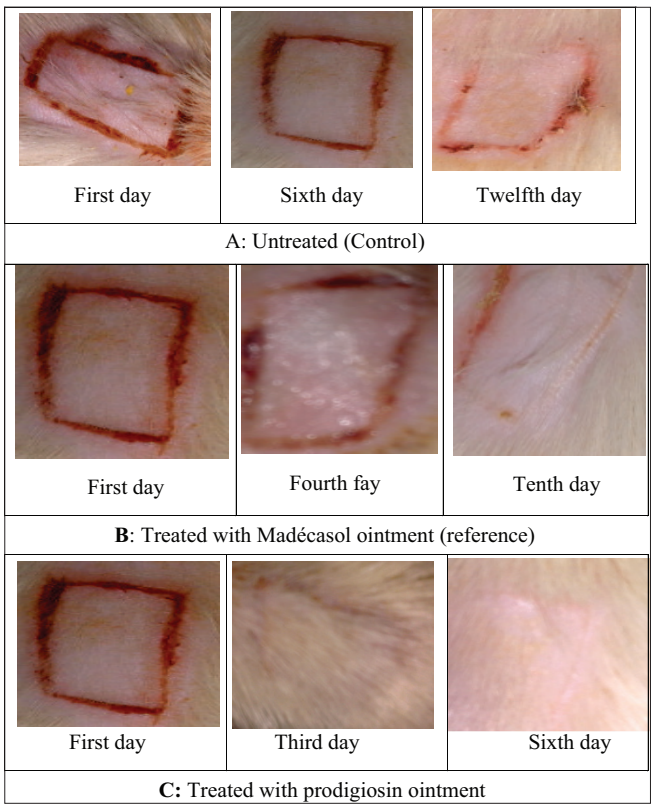

Fig. 8: Photographic representation of contraction rate showing wound contraction area on different post excision days.

prodigiosin was studied by estimating free radicals scavenging assay, β carotene discoloration, and FRAP. Few data were found reporting the antioxidant capacity of prodigiosin from *S. marcescens* by those methods. The investigation showed higher antioxidant activity with the DPPH assay, which could be related to the characteristics of this method since DPPH provided information on the reactivity of test compounds with a stable free radical [20]. The assay measures the reducing ability of antioxidants against the oxidative effects of ROS. Free radical scavenging molecules exert both anti-inflammatory and antibacterial effect [21,22]. Here, our finding revealed that treatment with ointment-based prodigiosin contributes to the increase in wound contraction. We observed completed cutaneous healing in 6 days when topical of prodigiosin was applicate. Anti-inflammatory activity is necessary for shorten the healing period [23]. Any agent that accelerates the wound healing process can be termed as a promoter of wound healing. The availability of substances capable of stimulating the process of would repair is still needed.

CONCLUSION

Prodigiosin showed effective antioxidative activities and a good susceptibility against all pathogenic bacteria and fungi. Having powerful wound healing potential. A based cream of prodigiosin accelerates wound healing activity in excised wound model, without a red phase, with the total disappearance of lesions. It is promising as a natural replacement for the synthetic drug. However, further works are needed to evaluate the healing activity at the tissue level, including tissue remodeling, re-epithelization, and collagen deposition.

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AUTHORS' CONTRIBUTIONS

The author has contributed to reviewing the preparation and editing of the manuscript.

CONFLICTS OF INTEREST

The author declares no conflicts of interest and is responsible for the content and writing of this article.

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