ABSTRACT

Objective: The aim of this work is to evaluate antioxidative, antimicrobial, and healing wound potential of prodigiosin extracted from *Serratia marcescens* strain microbota 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). *C. 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*.

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 therapeutical 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 prodigiosin 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 tripyrrole 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, antifungal, antibacterial, anti-protozoa, and antimalarial 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 Merieux) 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-30, 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/min) 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 infrared 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 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 (IC₅₀) 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 antioxidative activity after 48 h was estimated according to the following relation:

RAA = Abs sample (48 h) / Abs BHT (0 h) × 100

RAA: Relative antioxidative 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 IC₅₀ 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 deaired. Incision wounds of about 2 cm² 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 = [(Md1 - Md0) / Md1] × 100

Md1: Measure 1+ day, Dn: Measure n+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 IC₅₀ 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 growth and prodigiosin production
As presented in Fig. 2, results suggested that the production of prodigiosin from Serratia marcescens BRI 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 growth dependent.

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

In _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. 1: MALDI-TOF MS spectrum for _Serratia marcescens_ BR1 strain](image)

![Fig. 2: Growth and prodigiosin production by _Serratia marcescens_ BR1 in LB broth at 30°C agitated rate of 160 rpm](image)

![Table 1: Antimicrobial activity of the prodigiosin extracts from _Serratia marcescens_ BR1](image)

| 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 |
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 applied. Anti-inflammatory activity is necessary for shortening 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 wound 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 prodigiosin contributes to the increase in wound contraction. The healing process can be termed as a promoter of wound healing. The availability of substances capable of stimulating the process of wound repair is still needed.

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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.

**REFERENCES**

1. Ansari MA, Jadona NS, Singh SP, Kumar A, Singh H. Effects of approaches to wound management. Clin Microbiol Rev 1997;14:244-69.
2. Sumathi C, Priya DM, Swarnalatha S, Dinesh MG, Sekaran G. Production of prodigiosin using tannery fleshing and evaluating its pharmacological effects. Scientific World Journal 2014;2014:290327.
3. Logeeswari K, Shubashini KS. Wound healing medicinal plants: A review. Int J Chem Environ Pharm Res 2012;39:199-218.
4. Lauro GJ. A primer on natural colours. Cereal Foods World 1991;36:949-53.
5. Chidambaram KV, Perumalsamy LP. An insightful overview on microbial pigment, prodigiosin. Electron J Biol 2009;5:49-61.
6. Raisaïnen R, Nousiainen P, Hynninen PH, Dermorubin and 5-chlorodermorubin. Textile Res J 2002,72:973-6.
7. Cang S, Sanada M, Jokho O, Ohta S, Nagamatsu Y, Yoshimoto A. High production of prodigiosin by *Serratia marcescens* grown on ethanol. Biotechnol Lett 2000;22:1761-5.
8. Furstner A. Chemistry and biology of roseophilin and the prodigiosin alkaloids: A survey of the last 2500 years. Chem Int Ed Engl 2003;42:3582-603.
9. Rémi P. Vers la synthèse d’analogues de la Prodigiosine. Synthèse de 2,2-bipyroles Dissymétriques. France: Thèse Docteur de L’Université de Grenoble; 2006.
10. Eric J, Kalivoda NA, Stella MA, Aston JE, Fender PP, Thompson R. Cyclic AMP negatively regulates prodigiosin production by *Serratia marcescens*. Res Microbiol 2010;161:158-67.
11. Ramani D, Nair A, Kritthika K. Optimization of cultural conditions for the production of prodigiosin by *Serratia marcescens* and screening for the antimicrobial activity of prodigiosin. Int J Pharmacoal Biol Sci 2014;5:383-92.
12. Namazkar S, Garg R, Ahmad WZ, Nordin N. Production and characterization of crude and encapsulated prodigiosin pigment. Int J Chem Sci App 2013;3:116-29.
13. Bharmal H, Jahagirdar N, Arun K. Study on optimization of prodigiosin production by *Serratia marcescens* MSK1 isolated from air. Int J Adv Biotech Res 2012;2:671-80.
14. Bertoncelj J, Dobersek I, Jammik M, Golob T. Evaluation of the phenolic content, antioxidant activity and colour of Slovenian honey. Food Chem 2007;105:822-8.
15. Athannena S, Chalghem I, Kassah-Louar A, Laroui S, Khebri S. Activité antioxydante, anti-inflammatoire et antimicrobienne d’extraits de *Cuminum cyminum* L. J Pharm Pharmacol 2002;54:365-71.
16. Oyaizu M. Studies on products of browning reactions: Antioxidative activities of product of browning reaction prepared from glucosamine. Japan J Nutr 1986;44:307-15.
17. Darah I, nazari TF, Kassim J, Lim SH. Prodigiosin-an antibacterial red pigment produced by *Serratia marcescens* IBRL USM 84 associated with a marine sponge *Xestospongia testudinaria*. J Appl Pharm Sci 2014;4:1-6.
18. Mosquera OM, Yaned MC, Jaime N. Antioxidant activity of plant extracts from Colombian flora. Braz J Pharmacoen 2009;19:382-7.
19. Sasaki YE, Kawaguchi S, Kamaya A, Ohbata M, Kanasawa K, Iwama K, et al. The comet assay with 8 mouse organs: Results with 39 currently used food additives. Mutat Res 2002;519:103-9.
20. Szabo MR, Idoiou C, Chamber D, Lupea AX. Improved DPPH determination for antioxidant activity spectrophotometric assay. Chem Pap 2007;61:214-6.
21. Sala A, Recio M, Giner RM, Manze S, Tournier H, Schinella G, et al. Anti-inflammatory and antioxidant properties of *Helichrysum italicum*. J Pharm Pharmacol 2002;54:363-71.
22. Conforti F, Sosa S, Marrelli M, Menichini F, Giancarlo AS, Uzunov D, et al. *In vivo* anti-inflammatory and *in vitro* antioxidant activities of Mediterranean dietary plants. J Ethnopharmacol 2008;116:144-51.
23. Shimizu N, Watanabe T, Arakawa T, Fujwara Y, Higuchi K, Kuroki T. Pentoxifylline accelerates gastric ulcer healing in rats: Roles of tumor necrosis factor alpha and neutrophils during the early phase of ulcer healing. Digestion 2000;61:157-64.