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Original article

Wound healing Activities of the bioactive compounds from *Micrococcus* sp. OUS9 isolated from marine water

K. Shanthi Kumari [a,b], Pabba Shivakrishna [c], V.S. Ramakrishna Ganduri [a,*]

[a] Department of Biotechnology, Koneru Lakshmaiah Education Foundation, Vaddeswaram, Guntur 522502, A.P., India
[b] Department of Microbiology, Osmania University, Hyderabad 500 007, Telangana, India
[c] Lavin Laboratories, Nallakunta, Hyderabad 500 044, Telangana, India

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ABSTRACT

Marine species are increasingly important as a source of specific biological active metabolites. Marine species comprise almost half of global biodiversity. Oceans and sea are thus the biggest source of positive natural compounds that could be utilized in the pharmaceutical industry as functional constituents. In the present study was to find out the wound healing property of the bioactive compounds from *Micrococcus sp*. OUS9 isolated from marine source. The in vivo wound healing activity was studied using excision wound model. The KLUF 10 and KLUF13 ointment was prepared and used to determine wound healing activity in albino rats. Topical application of the ointment enhanced the contraction of wound in contrast with rat control group. KLUF13 had shown strong healing ability in wounds and had a positive influence on the various phases of wound repair.

1. Introduction

Wounds are physical injuries which damage or separate the skin. Proper cure is important for wound repair, which is necessary for anatomical stability and skin disruption. Tissue repair is a series of inflammation, migration and proliferation, sequences of different cell types (Sidhu et al., 1999). Equally the stage of inflammation starts immediately after injury, first with the vasoconstriction that promotes homeostasis and releases mediators of inflammation. The proliferative process is characterized by the proliferation of granulation tissue which is primarily produced by fibroblast and angiogenesis. The remodeling stage is characterized by reformulations and improvements in the collagen fiber components which increase the strength of the tensile (Varoglu et al., 2010).

Repeated trauma, inadequate perfusion or oxygenation and excessive inflammation are factor that contribute to causing and perpetuating the chronicity of wounds (Harding et al., 2005). Imbalance has been observed in free radical generations and antioxidants to cause tissue damage and oxidative stress, and belated wound healing. Elimination of ROS could therefore be a significant strategy to cure chronic wounds (Mikhal'chik et al., 2006). New drugs, such as cancer, microbial and inflammatory processes, are increasingly needed to treat human diseases, and new bioactive compounds are also being sought.

Marine species are considered the best source of production for such compounds. Marine species are seen as the greatest source of energy for such compounds. Several of these compounds have been identified as having different biological activities: anticancer, anticoagulants and anti-hypercholesterolemic activity have been documented in fish-isolated peptides and algal polysaccharides (Lordan et al., 2011). All marine bacteria and fish oils have large amounts of fatty acids, while seaweeds and crustaceans have strong antioxidants, such as phenolic and carotenoid compounds (Rasmussen and Morrissey, 2007).

Marine microorganisms have been studied in the last 20 years to detect new medications and are considered as special biological reservoirs of active secondary metabolites (Hughes and Fenical, 2010; Blunt et al., 2016; Amedei et al., 2012). The Ocean is a rich source of diversity in bio-and chemical matters. Although this diversity is the source of specific chemical compounds, it remains underexplored its potential for pharmaceutical applications (Kijjoa and Sawangwong, 2004; Albericio et al., 2010). Marine microorganisms exhibit unique metabolic and physiological capacities that allow them to survive under extreme conditions and thus

* Corresponding author.
E-mail address: krishna.ganduri@kluniversity.in (V.S.R. Ganduri).

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produce new bioactive compounds that cannot be found elsewhere (De Carvalho and Fernandes, 2010; Satpute et al., 2010). The aims of the study are to analyze the wound healing function of bacterial extracts through excision, wound models on Wistar albino rats.

2. Materials and methods

2.1. Chemicals and experimental animals

All the chemicals and solvents used in the research study were analytical grade and ordered from Merck Millipore. Wistar Albino rats around 8–10 weeks (150–250 g) weight were used for the analysis (Jeeva Life Sciences, Hyderabad). The rats were fed a regular pellet diet and kept under standard suitable conditions in polypropylene cages (12 h light–dark cycle; 25 ± 3 °C; 35–60% RH). The rats were left to acclimatize for 10 days at room temperature. For each group a total of 6 rats were used. The experimental rats used, and the procedures followed in this research, were reviewed and accepted by the Committee on Institutional Animal Ethics (44/JLS/IAEC/18) before the experiment was started.

2.2. Isolation of marine bacteria

The intertidal environment at coastal locations in Nellore Krishnapatnam, India has been used to collect seawater samples. The samples were spread over the complete surface of Zobell agar plates consisting of ferric citrate $C_6H_5FeO_7$: 0.1, g/L, peptone: 5.0 g/L, yeast extract: 1.0 g/L, sodium sulphate Na_2SO_4: 3.24 g/L, sodium chloride NaCl: 19.45 g/L, magnesium chloride $MgCl_2$: 8.8 g/L, potassium chloride KCl: 0.55 g/L, calcium chloride $CaCl_2$: 1.8 g/L, sodium bicarbonate $NaHCO_3$: 0.16 g/L, strontium chloride $SrCl_2$: 0.034 g/L, boric acid H_3BO_3: 0.022 g/L, potassium bromide KBr: 0.08 g/L, sodium silicate Na_2SiO_3: 0.004 g/L, ammonium nitrate NH_4NO_3: 0.0016 g/L, sodium fluoride NaF: 0.0024 g/L, disodium phosphate Na_2HPO_4: 0.008 g/L, agar: 15.0 g/L. All colonies were screened after incubation at 25° C for 48 h and those with different pigmented bacteria and morphology were selected.

2.3. Molecular-based characterization

By using 16 s rRNA sequencing, the bacterial strain that showed the best inhibition against the selected pathogens was subjected to molecular identification. The bacterial culture DNA sample, which was amplified using the 16S rRNA primers main forward (5′-TCACGGAGTTT-GATCCTG-3) and reverse (5′-GCGGCTGCACGTA GTT-3′) primers, was then sequenced to generate 16S rRNA gene sequence. The resulting sequence was then evaluated using the BLAST similarity search program and contrasted with nucleotide in the "GenBank" (NCBI) database (Krishna et al., 2015). A phylogenetic tree was acquired with maximum probability demonstrating the evolutionary relationships between the chosen sequences (Giovannoni et al., 1990).

2.4. Fermentation process

The fermentation was performed using 250 ml capacity Erlenmeyer flasks for the selected active bacterial strains; contain 100 ml of Zobell medium. The pure selected bacteria were inoculated with 1 ml culture suspension for the sterilized fermented broth. On an orbital shaking incubator at 250 rpm, inoculated flasks were incubated at 28 °C for 5 days. The fermented media was centrifuged for crude extract preparation at 10,000 rpm or 20 min after incubation.

2.5. Extraction of crude bioactive compounds

Crude bioactive compounds were extracted for pigment as per Slater et al. (2003). In short, 10 ml of the culture 3 was separated from the broth and centrifuged for 10 min at 10,000*g*. Extraction of pigments was done by applying methanol to the pellet and incubating at 60 °C for 20 min accompanied by centrifugation (10000*g*, 10 min).

2.6. Purification and characterization

The column chromatography had chosen with silica gel of 100–200 μm particle size. The gel was suspended for the packing of the column with petroleum ether. The column was formed by a corning glass tube 40 cm long with a glass stopper at the bottom and an internal diameter of 2.5 cm. The column's final size was 25 × 2.5 cm. The column had methanol balanced. The sample was not exceeding 5 ml, and the flow rate was kept to 0.2 ml/min, with the chloroform gradient water system methanol (9:1, 7:3, 1:1). Eventually, methanol and hexane washed the base. 10 ml fractions were gathered and all the different fractions were analyzed with each solvent scheme. All pooled fractions have been tested with antimicrobial agents. The active fraction was analyzed and characterized using NMR and Mass spectroscopy.

2.7. In vitro (Scratch assay) wound healing activity

In vitro cell migration of L929 cells by a process already reported (Liang et al., 2007). 2×10^5 cells/mL were seeded and cultivated overnight in 6-well plates. Delbucco's Phosphate Buffered saline (DPBS) washed cells and a 200 μL sterile tip was scratched. After thorough washing the cells with DPBS, the detached cells and other cellular debris were eliminated. The cells have been treated with A of 125 μg/mL. Two pure fractions and positive monitoring 25 μg/mL and 24-hour incubation. Cipladine is a standard drug used in the treatment of wounds (Kumar et al., 2007; James and Friday, 2010). There was negative control of untreated cells. In the images taken by inverted microscope, fitted with digital camera, cell migration and morphological changes of cells were observed. The triplicate tests were performed (n = 3). The scratch and wound closure distance at different intervals of time (0, 12, 24 and 48hrs).

2.8. Preparation of ointment

The extract was formulated in the form of ointment for assessment of wound healing operation by excision wound model. The ointment is prepared by fusion process. The materials used for the preparation of simple ointment include wool fat, white soft paraffin, cetosteryl alcohol, and hard paraffin, which were heated as to the increasing order of their melting point and gently mixed with stirring followed by cooling and packing in wide mouth. Likewise, 10 percent ointment was made from two pure fractions.

2.9. In vivo wound healing activity

The model of spherical excision wound was used to determine the contraction of wound and the duration of closure of wound. Each animal category was anaesthetized. After the application area was shaved, a circular wound was created on each rat's dorsal interscapular region by excising the skin with a 5 mm and leaving the wounds open (Tramontina et al., 2002). The test samples, standard drug and vehicle ointments were applied topically once a day until the wound was healed completely.

2.10. Dosage

In male Wistar Albino rats the effect of two pure fractions on wound healing activity was investigated. Total 24 numbers of animals weighing 150–200 g was selected randomly and divided into 4 groups composed of 6 rats in each group separate study groups (6 rats in each group):

Group I : Control group without treatment
Group II : marketed drug treated with 2% Framycetin ointment
Group III : Test group Treated with KLUF-10 fractions ointment
Group IV : Test group Treated with KLUF-13 fractions ointment

2.11. Wound index measurement and Skin irritation studies

The wound indices were calculated in a random scoring system every 2 days after wound formation. The healing activity was calculated as a % of wound contraction, using automated calipers to measure the duration and size of the wound following the formula (Walker and Mason Jr, 1968). Importance in the wound healing test group was achieved by contrasting the healed wound area on the respective days with the healed wound region of the control group. This study was done to test for any skin discomfort in the animal model. On the rat's dorsal side three sites have been identified This study was carried out to test the animal model for any skin irritation. Trois sites have been found on the dorsal side of the rodent.

3. Results and discussion

In the present investigation the total 29 different bacterial cultures were isolated from different locations of Nellore district regions the bacterial colonies present on agar plates with morphologically different pigment producing has been identified. The Selected colonies were subcultured in individual test-tube and observed for morphological characterization. The chosen colonies were screened by well diffusion method for antagonistic activity. process against two pathogenic bacteria out of 29 selected bacterial cultures OUS9 have shown efficient antagonistic activity, the results showed that the selected isolate have good growth and ability of high pigment producing capacity. The strain was further checked by ribotyping molecular technique using gene sequence of 16 s rRNA, which was amplified further sequenced. The partial sequence of the 16S rRNA gene obtained was submitted to BLAST and found to be *Micrococcus* sp. OUS9 (Fig. 2) the sequence is deposited in the Genbank (Accession number MN108086). Microorganisms living in the rhizosphers of diversity of crops are probable, because of the abundance of substrate exuded from the roots compared with non-Rhizosphere soils, to synthesize and release auxin as secondary metabolites (Ahmad et al., 2005).

Isolation of crude secondary metabolites by solvent extraction is very significant phenomenon, to find a good suitable solvent that have the prospective to extract high yield and most effective bioactive compounds. Research has shown the strong antimicrobial potential of ethyl acetate extract against the pathogens of fungi and bacteria (Kobayashi et al., 2004). Here from the eluted fractions with column chromatography is tested for antimicrobial study and the best fraction was further identified. KLUF-10 and KLUF-13 with molecular weight 568.9 and KLUF-13 the molecular weight with 263.38. The purified compound is proposed on the basis of these observations KLUF-10 as 3-Hydroxy-β, ε-caroten-3′-one, KLUF-13 1-(1-(4-methoxphyenyl)-2-(methyl amino) ethyl) cyclohexan-1-ol and which was isolated from *Micrococcus* sp. OUS9 for the first time and is to appear that novel compound with good antimicrobial activity. The most promising results for anticancer activity were obtained with KLUF-10 and KLUF-13 fractions of the *Micrococcus* sp. OUS9 strain.

Because of physical, thermal injuries or chemical wounds are normal in humans and animals (Barreto et al., 2014). Under management the degradation of the tissue frequently leads to chronic inflammation and secondary infections. Untreated wounds can

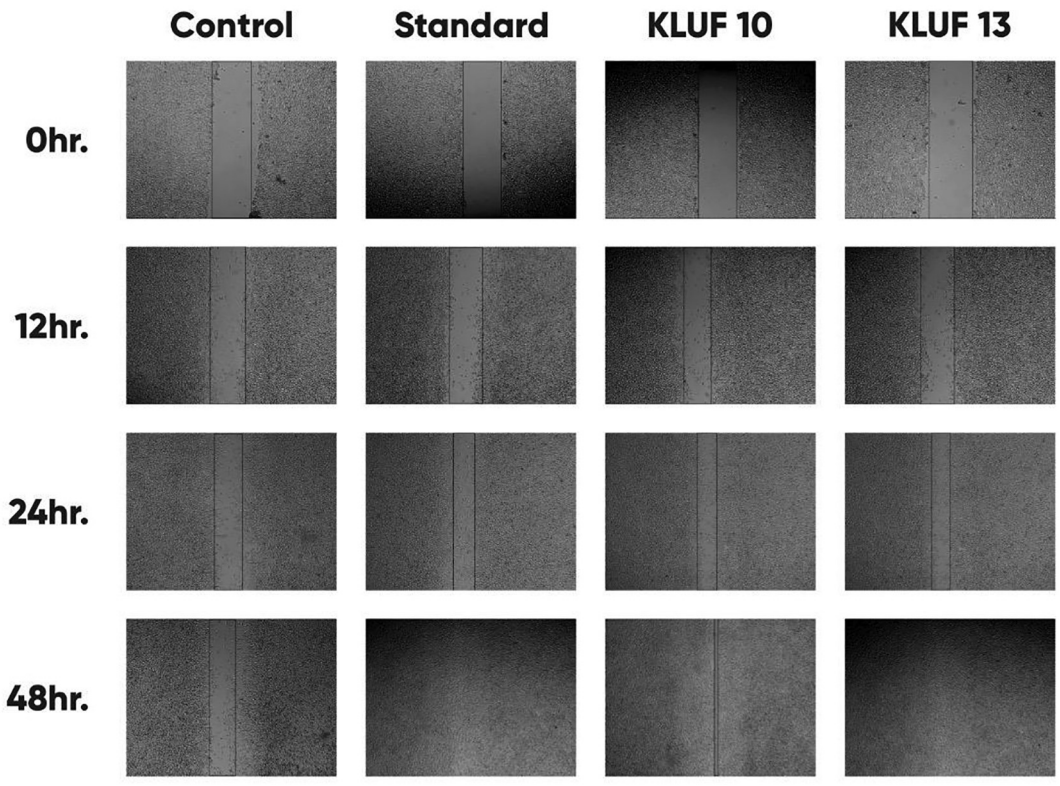

Fig. 1. *In-vitro* scratch wound healing test showing the migration of the L929 cell to the cell-free (outlined).

lead to substantial losses due to lower efficiency and death in animals of production. For wound care, there are common properties that natural remedies can possess, such as controlling/managing secondary infections and inflammation and encouraging tissue regeneration, among other healing properties. Damage associated with injury can include the epidermis, dermis, local vasculature, and probably other underlying tissues, and this typically activates various wound healing procedure. (Daunton et al., 2012).

Wound dressing with natural compounds is very important to prevent contaminants from entering the human body in order to avoid external bacterial contamination (Thomas et al., 1982). Different studies have indicated that the acceleration of wound healing and remediations for using chitin/chitosan or alginate in wound treatment has already been reported on the market (Ishihara et al., 2001; Obara et al., 2003; 2005) and alginate (Queen et al., 1987). The wound healing procedure is an *in vitro* standard method for the study of collective cell migration. Sheet migration is known as collective cell migration, as studied in a wound healing assay. Epithelial and endothelial monolayers are described in this migration, traveling in two dimensions while preserving their cellular connections. (Friedl and Wolf, 2010; Rørth, 2009; Vedula et al., 2013). Sheet migration occurs in several procedures including embryonic morphogenesis and tissue lesions (Pouliot et al., 2011; Fujisawa et al., 2012).

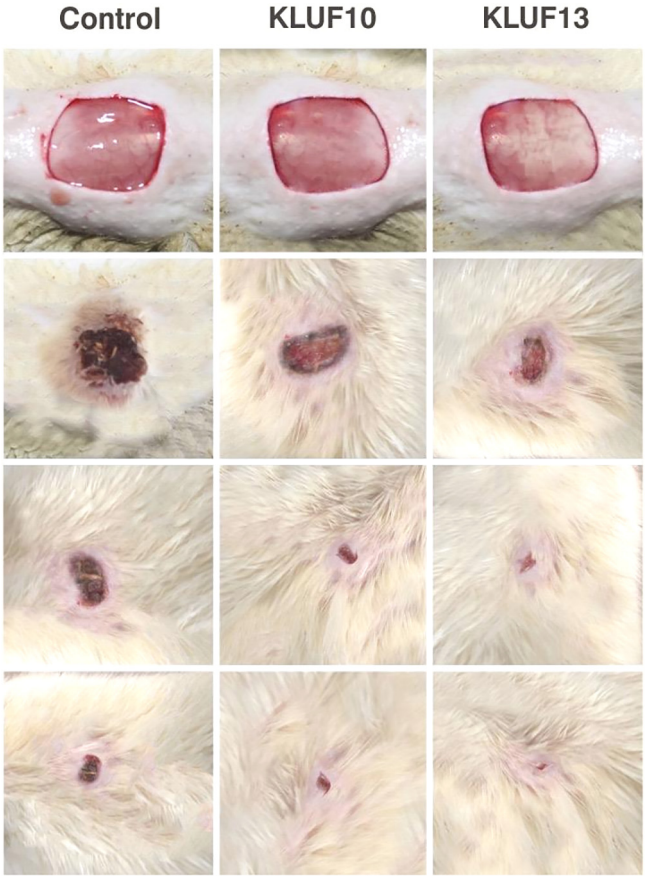

Fig. 2. Effect of the KLUF 10 and KLUF13 fraction on healing studies.

Further, Hu et al. (2017) reported in his investigations that the *In vitro* scratch assay and wound healing experiments of deep partial-thickness scald wound in rabbits indicated that MCPs from the skin of tilapia were an effective and promising agent for burn care. In addition, attempt of herbal extracts were effective in wound healing, through the improvement in the migration of fibroblast cells and regulating the gene expression of Tgfβ1 and Vegf-A genes in fibroblast cells treated with extracts (Negahdari et al., 2017). In similar studies by Yang et al. (2017) reported taht the therapeutic effect of *D. genkwa* on anal fistula and showed the extractive from *D. genkwa* root benefited the wound healing through up-regulation of collagen genes in HSFs. Later, plant extracts of *A. polystachyus* leaves have also demonstrated with wound healing activities and justified the use of these extracts for *S. aureus* infected wounds (Demilew et al., 2018). And, *Hibiscus hirtus* ethanolic extracts also had proven excellent antimicrobial activity towards wound healing activity (Ravishankar et al., 2018).

In the present research study, L929 cells were treated with 25 µg/mL of KLUF 10 and KLUF13 fractions that are isolated from *Micrococcus* sp. OUS9 for 48hrs. The cell migration was observed at 0, 12, 24 and 48 h and the microscope wound closers time. The results showed that at 25 µg/mL, the KLUF-10 fraction closed the gap created by the scratch in 48 h by 96.29%. And 94.362% followed with KLUF-16 fraction (Fig. 1). The microscopic images of marketed drug-treated, untreated and extract-treated L929 cells show 99.05 percent of the gap at 48 h in the standard drug-treated cells. The use of herbal remedies has increased exponentially in recent years. Their relatively low cost, natural origin and less adverse effects are making these agents more common in developed and developing countries (Modak, et al., 2007). Initially plant derivatives were used to use numerous drugs used in conventional medicine (Oreagba et al., 2011). In the recent study, Shiva et al. (2019) confirmed faster reduction in wound area from red pigment in comparison with control and framycetin ointment treated groups and indicated that red pigment shown significant wound healing potential with positive influence on wound repair phases.

In this analysis fractions of KLUF 10 and KLUF13 are isolated from Micrococcus sp. OUS9. In comparison to the control group of albino-rats and reference standard, Micrococcus sp. OUS9 showed notable wound healing prosperity (Fig. 2). In this experiment, wound-healing activity of the KLUF 10 and the KLUF13 were used at various levels of 100 and 200 mg/kg. The significant wound contraction was demonstrated in the 200 mg/kg concentration with both KLUF 10 and KLUF13 treatment groups. The wound contraction of KLUF 10 treated animals shown with 298.70 ± 6.84 and KLUF 13 with 264.02 ± 6.12 on 8th day (Table 1). However, KLUF 13 treatments showed significantly higher wound healing property as

Table 1
The influence on the percentage of wound contraction of the KLUF 10 and KLUF13 fraction formulated in the ointment.

Oral treatment	Dosage mg/kg	Percentage of contraction in wound area (mm^2/rat) after different days						
		0th day	4th day	8th day	12th day	14th day	18th day	20th day
Control	6%/w	432.8 ± 6.93	417.7 ± 6.77	312.3 ± 10.8	178.5 ± 10.1	103.3 ± 5.79	39.2 ± 1.25	11.3 ± 0.67
Standard	567	468 ± 1.6	382 ± 2.8	210 ± 1.8	198 ± 1.8	70 ± 1.8	4 ± 1.8	0 ± 1.8
KLUF 10	100	420.3 ± 7.15	359.2 ± 13.2	298.70 ± 6.84	94.07 ± 12.43	50.89 ± 10.02	9 ± 47.09	1.40 ± 1.29
	200	418.70 ± 6.84	301.21 ± 6.14	217.8 ± 1.8	73.2 ± 4.21	41.0 ± 3.86	4.80 ± 0.56	1.0 ± 0.0
KLUF 13	100	430.0 ± 7.02	361.7 ± 12.1	219.8 ± 14.2	67.3 ± 2.55	42.8 ± 6.13	4.83 ± 0.98	1.70 ± 1.18
	200	410.3 ± 7.15	301.21 ± 6.14	264.02 ± 6.12	64.89 ± 8.17	24.08 ± 5.39	3.40 ± 1.29	0.0 ± 0.0

compared to the KLUF 10 and with standard and control after 20 days.

4. Conclusion

This report clearly demonstrated that KLUF 10 and KLUF13 fraction was effective in enriching wound closure progression in L929 cells and in vivo wound contraction. Therefore, it may be potential candidate based on natural products to elucidate the problem of delayed wound healing among the patients. The bioactive metabolites present in this crude extract have been successfully identified and validated by NMR studies as zeaxanthin and 1-(1-(4-methoxy phenyl)-2-(methylamino)ethyl)cyclohexanol, with the corresponding *in-vivo* activities have also demonstrated remarkable support for *Micrococcus* sp. OUS9 towards wound healing potential. In summary, the findings of the research suggest a new understanding of the use of wound healing compounds and potential injury healing and could be a viable source for obtaining natural wound healing compounds.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Further Reading

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