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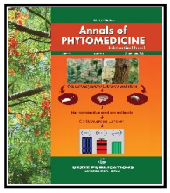

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Antimicrobial and wound healing potential of fungal pigments from *Thermomyces* sp. and *Penicillium purpurogenum* in wistar rats

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Abstract

This study was conducted to evaluate the wound healing activity of pigments from methanolic extracts of *Thermomyces* sp. and *Penicillium purpurogenum*. The wound healing properties were determined using the excision wound model. The methanolic extract of fungal pigments showed antimicrobial activity against *Staphylococous aureus*. Excision wounds contaminated with *S. aureus* and subsequently treated with fungal pigment showed accelerated wound healing, the highest percentage wound contraction and maximum rate of epithelialization. Two per cent yellow pigment from *Thermomyces* sp. showed accelerated wound healing compared to red pigment from *P. purpurogenum*. Fourteen days post treatment, assessment on viable bacterial count on excision wounds in pigment treated groups were significantly lower compared against the control. It is concluded that methanolic extract of 2% yellow pigment from *Thermomyces* sp. was capable of curing wound at faster rate when compared to control.

1. Introduction

Wound healing is a multiphased process that includes inflammation, wound contraction, re-epithelialization tissue, re-modeling, and the formation of granulation tissue with angiogenesis (Kumarasamyraja *et al*., 2012). Several causes, including bacterial infection, necrotic tissue and blood supply interference, lymphatic obstruction, and diabetes mellitus, slow or prevent wound healing.

Bacterial infection is one of the extrinsic factors that can significantly impede wound healing. As a result, any treatment that protects the area against bacterial infection might speed up the healing process. Human pathogenic bacteria, on the other hand, have acquired resistance mechanisms to widely accessible antibiotics in recent years (Immanuel *et al*., 2012; Shiva Krishna *et al*., 2019).

Natural remedies have been exploited in folk medicine for thousands of years and are advised for a variety of diseases.

Antibiotics have been discovered because of bacteria and fungus found in the soil. Eczema, inflammation, superficial skin diseases, and wounds are all treated with dirt by Arab tribes in the Middle East. The filamentous Actinomycetales are known for their capacity to create a vast variety of chemically diverse secondary metabolites.

Members of the fungus and actinomycetales families continue to be prolific producers of new secondary metabolites with a variety of biological functions that might be used as anti-infection, anti-cancer, or other pharmaceutically beneficial substances in the future (Eteghad *et al*., 2013).

Microbial secondary metabolites are rich sources of therapeutically useful compounds. Fungal metabolites proved to be the most widely used in biotechnology as it produces of various compounds, including organic acids, vitamins, pigments and antibiotics. Though, many microorganisms produce colours, only few could be commercially exploited. The important fungal pigment-producing organisms are *Monascus purpureus, Monascus pilosus, Paecilomyces sinclarii, Epicocoum nigrum, Penicillium purpurogenum, Blackslea trispora* and *Aspergillus carbonarious.* (Gomez and Nosanchuk, 2001; Csehati, 2006). *Alternaria alternata, Wangiella dermatidis* and *Aspergillus nidulans* producing melanin pigments which protects melanocytes and keartinocytes prevents DNA break down by hydrogen peroxide, indicating pigment has antioxidant role in skin cancer (Joshi, 2003).

Pigment from endophytic fungus proved to be more potent than the commercially available antibiotic Streptomycin. It was effective against bacteria like *Klebsiella pneumoniae, staphylococcus aureus, Salmonella typhi* and *Vibrio cholera* (Visalakchi *et al.*, 2010). This present paper focuses on the wound healing potential of *Thermomyces* sp. and *P. purpurogenum* pigment in wistar rats.

2. Materials and Methods

2.1 Preparation of fungal pigment

Three extracellular pigment producing fungi, namely; *Penicillium purpurogenum, Chaetomium* sp. and *Thermomyces* sp., were isolated from soil. The two fungal cultures *Monascus purpureus* and

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Monascus ruber and human pathogens used in the study were obtained from MTCC, Chandigarh as reference culture. The fungal cultures grown on potato dextrose broth were incubated at 35ºC for 5-7 days. Supernatant were extracted with methanol filtered through Whatman No.1 filter paper and concentrated using vacuum rotary evaporator. The concentrates were then lyophilized and stored at –20ºC until they were utilized for assays. The dried extract was used directly for analyses of antimicrobial activity. The pigment diluted to 1% and 2% concentration for further uses.

The human pathogens (bacteria and fungi) *S. aureus, Bacillus. subtilis, Escherichia coli, Salmonella typhi and Candida albicans* used in the study were obtained from the IMTECH culture collection centre, Chandigarh, India

2.2 Determination of *in vitro* antimicrobial activity of fungal pigments

The human pathogens (bacteria and fungi) was multiplied in nutrient broth at 37°C for 18-24 h. A load of 10^8- 10^9 cfu/ml. 0.1 ml of culture broth was spread on nutrient agar plate by spread plate method. A well was formed using sterile cork borer. One hundred microlitre (µl) of pigment was poured in the well. At the end of 24 h incubation at 37ºC, the susceptibility of the organisms was determined by measuring the clear zone around the well (Iqbal *et al.*, 1998).

2.3 Experimental animals, housing conditions and diet

Healthy wistar albino rats weighing about 150-250 g each of either sex, were used for the study. Animals were obtained from KMCH College and Pharmacy, Coimbatore, Tamil Nadu and maintained in the Institute Animal House Facility. Animals were acclimatized for a week into 6 groups, comprising of 7 animals of each type (M/F). All animals were housed in polycarbonate cages on soft chip bedding, changed twice per week at room temp., 25 ± 2°C; relative humidity, 60-70%. Tap water was provided for drinking. They were exposed to a light and dark cycle of 12 h duration and fed with commercial diet (Ms/ Amrut - laboratory animal feed, Pranav Agro Industrial Ltd.) obtained from Bangalore. The experimental protocol was subjected to the scrutiny of the Institutional Animal Ethics Committee (CPCSEA Registration No- 685/Po/02/a/CPCSEA-2013) and was cleared by same before beginning the experiment.

2.4 Wound healing studies : Creation and contamination of excision wound

Ten per cent of the body furs were clipped from about surface 24 h before the experiment. The clipped portion was infected, with 0.1 ml of *Staphylococcus aureus* suspension (2×10^8 CFU/ml) prepared from an overnight culture (Kugelberg *et al.*, 2005). An excision wound model was made on the dorsal region. Infected mice were divided into six groups of five animals each (three control and three test groups). The first group was control, the second received vehicle and third received gentamycin (2%). The four other groups were treated with yellow and red pigment extract at 1 and 2% (w/v), respectively. Once in a day, 0.05 g of pigment extract was applied as dermal application for 14 days 48 h after of the infection. During the experiment period, body weights were measured every day (Mori *et al.*, 2002).

2.5 Percentage wound contraction

The wound diameter of each animal was measured at 3, 5, 7, 9, 11, 13, and 15 days post wounding (DPW), using a transparent meter rule, and the percentage wound contraction calculated using the method of Ezike *et al*. (2010).

2.6 Organ weight and histopathological studies

Vital organs of each rats such as liver, kidney, heart, spleen and lungs were excised and weighed. A section of the granuloma tissue was fixed in 10 % formalin for 3 days. These tissues were sliced in pieces, processed; stained using hematoxylin, eosin reagent and diphenyl xylene mounting fluid. The stained specimens were mounted on glass slides and observed under power microscope for determination of the pattern of lay-down for collagen.

2.7 Determination of wound microbial load

Skin sample was excised from each treatment from chloroform anesthetised animals. The skin sample was homogenized in normal saline. Using a sterile pipette, 0.1 ml of the 10^{-2} dilution was introduced on the surface of sterile mannitol salt agar medium and distribution with a sterile glass spreader. The inoculated plates were incubated at 37°C for 24 h and the colonies were counted

2.8 Statistical analysis

Mean and standard error were calculated using one-way ANOVA. Values of $p<0.05$ were considered statistically significant.

3. Results

Yellow pigment from *Thermomyces* sp. was found to be effective in inhibiting *S. aureus* (2.5 cm) and *B. subtilis* (1.5 cm) and *C. albicans* (1.64 cm) Red fungal pigments from *P. purpurogenum* exhibit excellent inhibitory effect against human pathogens, *viz* ., *E. coli* (1.34 cm) and *S. typhi* (1.6 cm) (Table 1.) Based on the intensity of inhibition produced by *Thermomyces* sp. was higher when compared to other pigments, it was selected for wound healing study.

Animals infected with *S. aureus* showed visible inflammation 48 h later, characterized by redness and swelling of the skin at the sites of inoculation. Treatment significantly reduced the bacterial concentration at the infection sites, with total suppression being noted with the 1 and 2% doses of yellow and red pigment as gentamycin in 5 days. Animals in all groups showed weight loss after infection. The study reveals that all the four groups showed decreased wound area from day-to-day. Yellow (1%) and red (2%) pigment treated animals showed a significant reduction in the wound area and epithelization period when compared to control. On 16th day, wound per cent area closure was control (83.59%), ciprofloxacin (97.3%), yellow pigment 1% (96.29%), yellow pigment 2% (97.53%), red pigment 1% (95.32%) and red pigment 2% (96.05%). In *Thermomyces* yellow pigment treated group, the period of epithelialisation was reduced to 13 day compared to 21 days in control, whereas red pigment treated group has taken 14.14 days (Table 2 and Figure 1).

From day 1 of treatment, body weight increased gradually with time. On the other hand, the weight gain by the animals of the negative control was lower than that of all the other groups. However, the weight gain by the animals that received the extract at 1 and 2% were comparable ($p>0.05$) to gentamycin group, and significantly higher ($p<0.05$) than control group (Table 3).

Table 1: Antimicrobial activity of fungal pigment extracts against human pathogens

Bacterial and fungal pathogens	Diameter of the inhibition zone in cm						
	Thermomyces sp.	*P. purpurogenum*	*Monascus purpureus*	*Monascus ruber*	Chaetomium sp.	Control	
						PC	NC
Gram positive							
S. aureus	2.5	2.1	1.1	0.85	NI	2.9	0.2
B. subtilis	1.5	0.93	0.74	0.4	0.6	2.1	0.0
Gram negative							
E. coli	1.24	1.34	1.28	0.3	0.5	2.8	0.3
S. typhi	1.37	1.61	0.84	NI	NI	3.2	0.0
Fungal pathogen							
C. albicans	1.64	1.21	1.24	NI	NI	2.0	0.0

PC = Positive control (chloramphenicol) ; NC = Negative control (methanol) ; NI = No inhibition

Table 2: Effect Thermomyces and Penicillium pigment on percent wound contraction in excision wound of rat

Experimental group	Epithelization period (days)	% of Wound contraction						
		3rd day	5th day	7th day	9th day	11th day	13th day	15th day
Wound only	21.54 ± 0.9	18.96 ± 0.88	37.78 ± 1.75	42.07 ± 1.9	50.19 ± 2.3	66.01 ± 3.05	77.19 ± 3.5	83.59 ± 3.8
Wound + *S. aureus*	23.78 ± 1.1	21.39 ± 0.99	41.27 ± 1.91	45.17 ± 2.0	59.45 ± 2.7	67.68 ± 3.1	75.32 ± 3.4	81.98 ± 3.7
Wound + *S. aureus* + ciprofloxacin	12.79 ± 0.59	40.41 ± 1.87	48.14 ± 2.22	58.02 ± 2.6	72.09 ± 3.3	80.78 ± 3.7	92.36 ± 4.2	97.03 ± 4.4
Wound + *S. aureus* + 1% yellow pigment	13.45 ± 0.62	25.01 ± 1.16	49.05 ± 2.29	61.57 ± 2.8	67.63 ± 3.12	85.64 ± 3.9	93.34 ± 4.3	96.29 ± 4.5
Wound + *S. aureus* + 2% yellow pigment	13.28 ± 0.61	27.05 ± 1.25	44.33 ± 2.05	62.06 ± 2.8	68.96 ± 3.19	82.51 ± 3.8	95.84 ± 4.4	97.53 ± 4.5
Wound + *S. aureus* + 1% red pigment	14.24 ± 0.66	28.57 ± 1.32	62.08 ± 2.9	67.24 ± 3.1	64.03 ± 2.9	80.29 ± 3.7	87.69 ± 4.05	95.32 ± 4.4
Wound + *S. aureus* + 2% red pigment	14.14 ± 0.65	31.77 ± 1.47	57.14 ± 2.6	66.99 ± 3.09	61.33 ± 2.8	86.69 ± 4.0	92.06 ± 4.2	96.05 ± 4.4

Table 3: Body weight of wistar rat fed with Thermomyces and Penicillium pigment for 28 days

Animals	Days of study				
	0	7	14	21	28
Male concentration (g/kg b.w)					
Wound only	124 ± 2.8	124 ± 2.8	124 ± 2.8	124 ± 2.8	124 ± 2.8
Wound + *S. aureus*	125 ± 2.8	125 ± 2.8	125 ± 2.8	125 ± 2.8	125 ± 2.8
Wound + *S. aureus* + ciprofloxacin	110 ± 2.5	110 ± 2.5	110 ± 2.5	110 ± 2.5	110 ± 2.5
Wound + *S. aureus* + 1% yellow pigment	134 ± 3.09	134 ± 3.09	134 ± 3.09	134 ± 3.09	134 ± 3.09
Wound + *S. aureus* + 2% yellow pigment	159 ± 3.6	159 ± 3.6	159 ± 3.6	159 ± 3.6	159 ± 3.6
Wound + *S. aureus* + 1% red pigment	112 ± 2.5	112 ± 2.5	112 ± 2.5	112 ± 2.5	112 ± 2.5
Wound + *S. aureus* + 2% red pigment	129 ± 2.9	129 ± 2.9	129 ± 2.9	129 ± 2.9	129 ± 2.9
Female concentration (g/kg b.w)					
Wound only	115 ± 1.7	124 ± 2.8	124 ± 2.8	124 ± 2.8	124 ± 2.8
Wound + *S. aureus*	126 ± 7.02	125 ± 2.8	125 ± 2.8	125 ± 2.8	125 ± 2.8
Wound + *S. aureus* + ciprofloxacin	095 ± 2.64	110 ± 2.5	110 ± 2.5	110 ± 2.5	110 ± 2.5
Wound + *S. aureus* + 1% yellow pigment	152 ± 1.2	134 ± 3.09	134 ± 3.09	134 ± 3.09	134 ± 3.09
Wound + *S. aureus* + 2% yellow pigment	114 ± 2.4	159 ± 3.6	159 ± 3.6	159 ± 3.6	159 ± 3.6
Wound + *S. aureus* + 1% red pigment	111 ± 2.5	112 ± 2.5	112 ± 2.5	112 ± 2.5	112 ± 2.5
Wound + *S. aureus* + 2% red pigment	122 ± 0.88	129 ± 2.9	129 ± 2.9	129 ± 2.9	129 ± 2.9

Data are mean ± SD of three measurements. $p<0.05$ compared to control

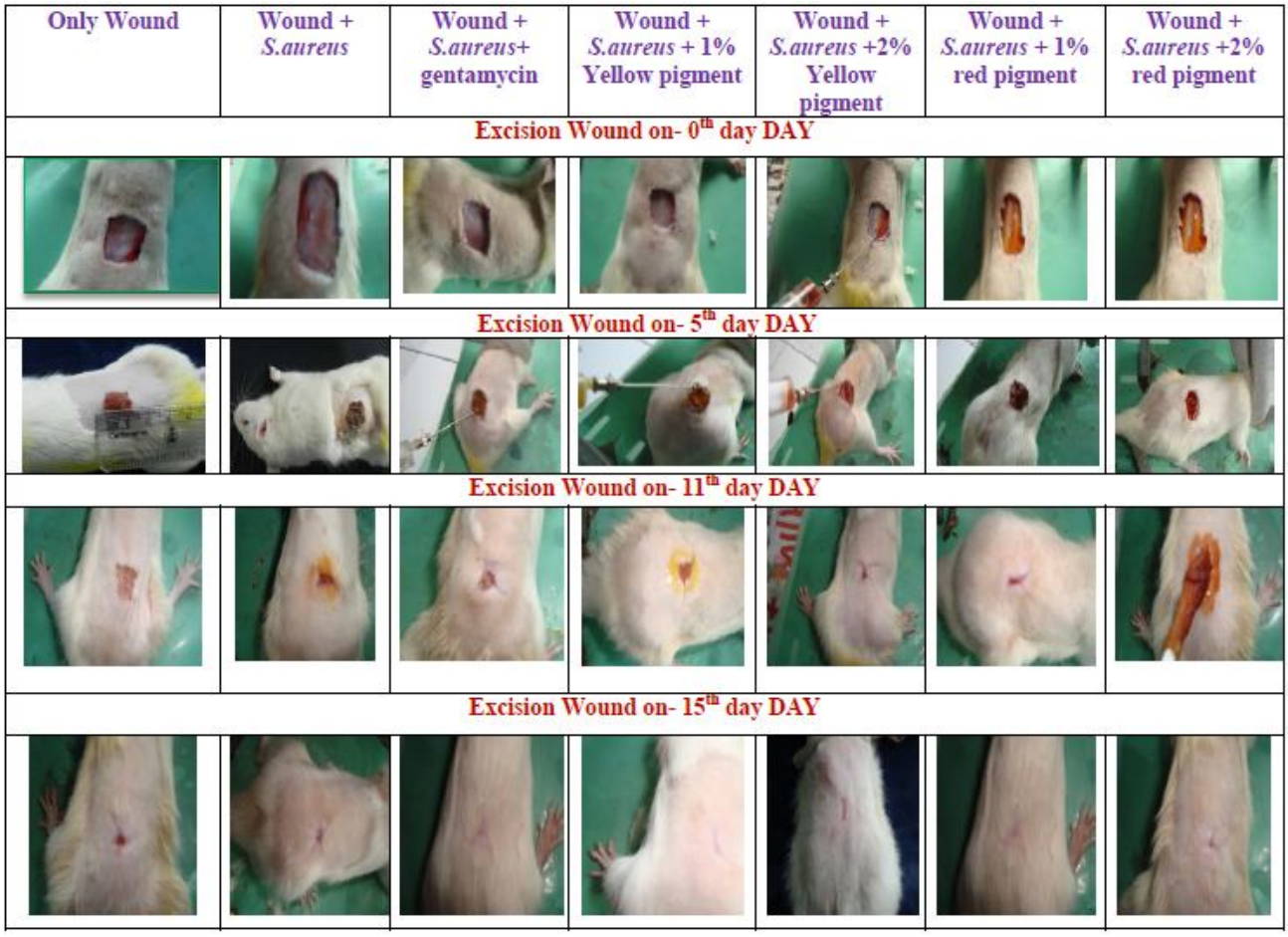

Figure 1: ***In vivo*** **antimicrobial and excision wound healing activity of fungal pigments.**

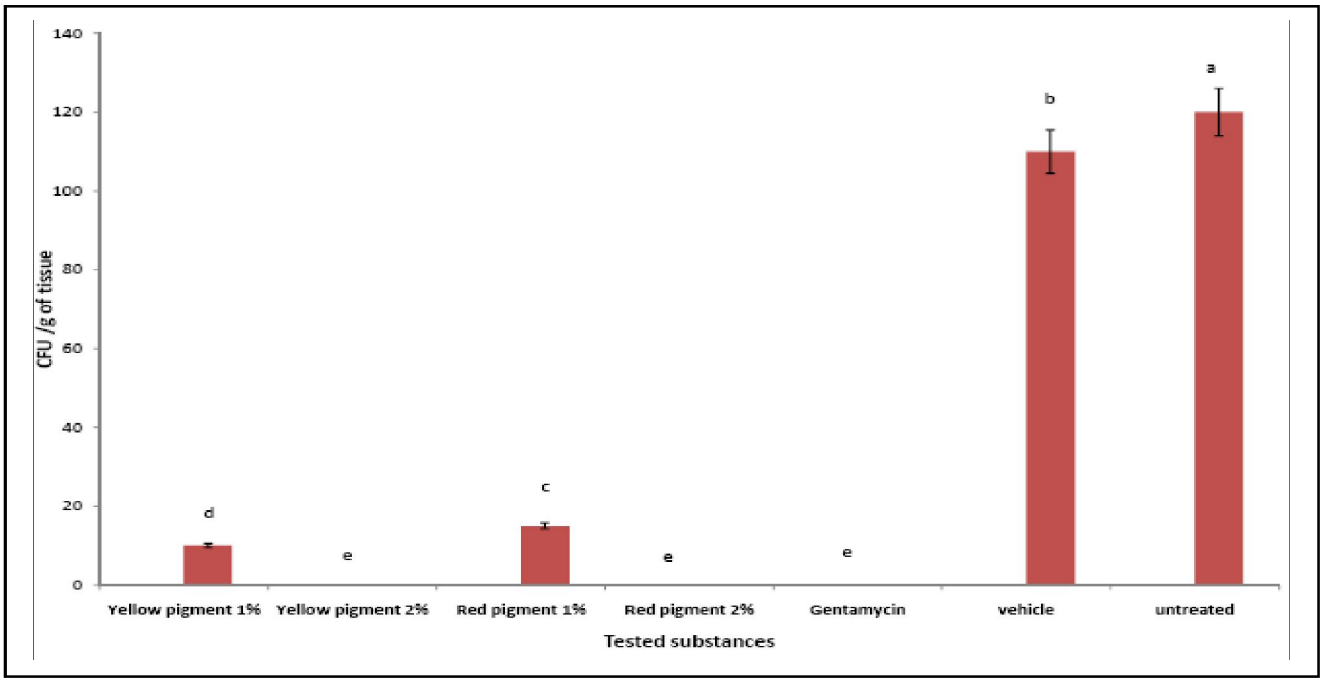

Figure 2: **Effect of fungal pigment on bacterial load on the 7th day of treatment.**

Table 4: Relative organ weight of rat fed with Thermomyces and Penicillium pigment

S.No.	Experimental group	Relative organ weights (g/animal)					
		Kidney (L)	Kidney (R)	Liver	Lungs	Heart	Spleen
Male							
1	Wound only	0.580± 0.01	0.64 ± 0.01	6.12 ± 0.01	1.41 ± 0.01	0.61 ± 0.01	0.85 ± 0.01
2	Wound + *S.aureus*	0.61 ± 0.03	0.68 ± 0.02	5.89 ± 0.01	2.14 ± 0.01	0.58 ± 0.01	0.91 ± 0.01
3	Wound + *S. aureus* + ciprofloxacin	0.58 ± 0.02	0.75 ± 0.04	5.12 ± 0.02	1.54 ± 0.01	0.07 ± 0.01	0.87 ± 0.02
4	Wound + *S. aureus* + 1% yellow pigment	0.64 ± 0.03	0.58 ± 0.02	5.09 ± 0.02	1.78 ± 0.01	0.75 ± 0.01	0.75 ± 0.02
5	Wound + *S. aureus* + 2% yellow pigment	0.68 ± 0.01	0.07 ± 0.02	6.04 ± 0.02	2.05 ± 0.01	0.78 ± 0.01	1.12 ± 0.02
6	Wound + *S. aureus* + 1% red pigment	0.45 ± 0.01	0.68 ± 0.04	5.45 ± 0.04	1.94 ± 0.01	0.63 ± 0.01	0.89 ± 0.02
7	Wound + *S. aureus* + 2% red pigment	0.69 ± 0.01	0.67 ± 0.04	5.36 ± 0.03	1.69 ± 0.01	0.75 ± 0.01	1.23 ± 0.02
Female							
1	Wound only	0.70 ± 0.05	0.76 ± 0.04	5.7 ± 0.03	1.35 ± 0.38	0.56 ± 0.01	0.86 ± 0.02
2	Wound + *S. aureus*	0.59 ± 0.03	0.61 ± 0.04	4.7 ± 0.24	1.54 ± 0.15	0.63 ± 0.03	0.99 ± 0.02
3	Wound + *S. aureus* + ciprofloxacin	0.56 ± 0.02	0.61 ± 0.02	4.9 ± 0.27	1.34 ± 0.03	0.66 ± 0.03	0.09 ± 0.02
4	Wound + *S. aureus* + 1% yellow pigment	0.66 ± 0.03	0.68 ± 0.04	5.3 ± 0.05	1.94 ± 0.11	0.78 ± 0.08	0.07 ± 0.04
5	Wound + *S. aureus* + 2% yellow pigment	0.60 ± 0.04	0.58 ± 0.08	4.4 ± 0.05	1.44 ± 0.11	0.51 ± 0.12	0.08 ± 0.04
6	Wound + *S. aureus* + 1% red pigment	0.60 ± 0.03	0.58 ± 0.02	5.3 ± 0.18	1.98 ± 0.29	0.53 ± 0.06	0.09 ± 0.04
7	Wound + *S. aureus* + 2% red pigment	0.60 ± 0.02	0.61 ± 0.02	4.7 ± 0.13	1.71 ± 0.17	0.65 ± 0.03	0.07 ± 0.04

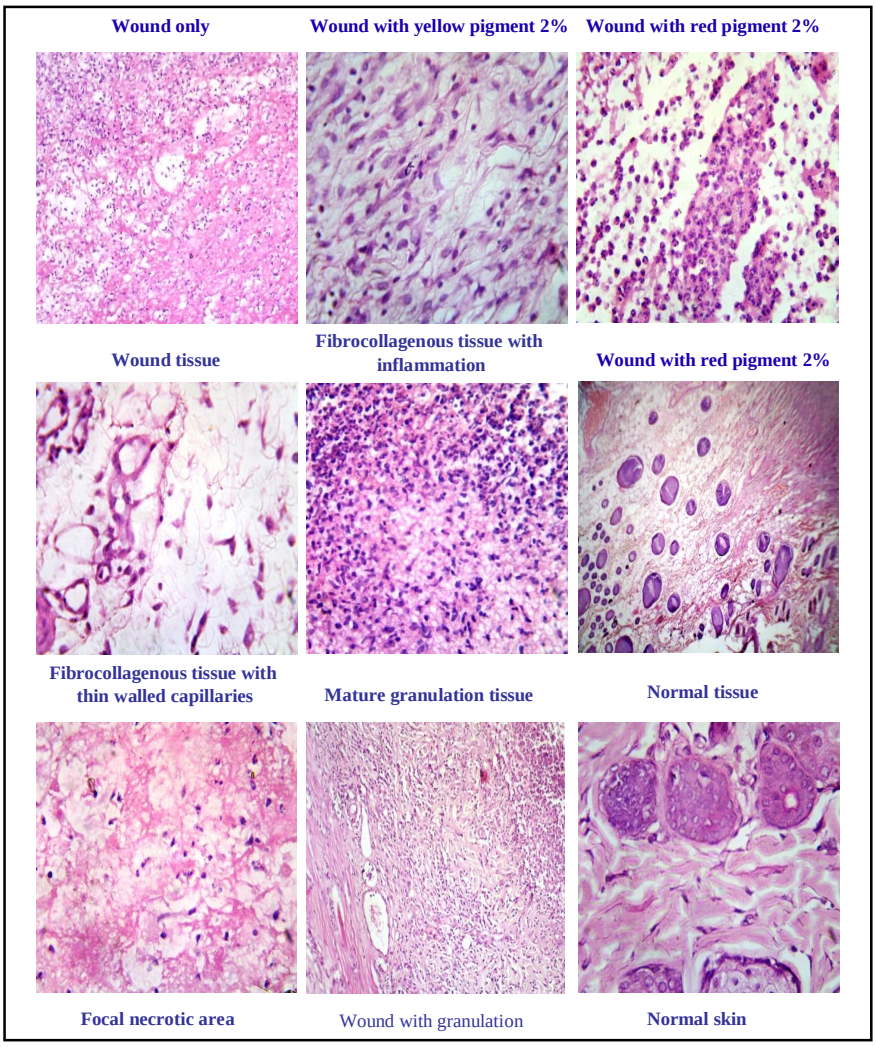

Figure 3: Histopathological observation showing the control sites and experimental sites on skin of the animal.

The significant decreases in *S. aureus* load in *Thermomyces* yellow pigment 1% treated groups. This suggests that the pigment extract has *in vivo* antibacterial effect against the tested microorganisms which are involved in wound contamination (Figure 2).

Organ to body weight ratio is one of the fundamental judgments to diagnose whether organ exposed to injury or not impaired organ often have abnormal timidity or atrophy (Table 3). There were no significant changes of organ to weight ratio in male and female rat or between treatment groups (Table 4).The histopathological observation showed increased granulation tissue and epithelial tissue in yellow and red pigment test groups as compared to control group (Figure 3).

4. Discussion

A promoter of wound healing is any substance that speeds up the healing of a wound. Despite enormous developments in the chemical industry, chemicals capable of boosting the would-repair process are still required. In folk medicine, several traditional plant-based medicines are recognised and utilised for treatment.

In reality, scientific investigations have shown that some of them do have biological effects on wound healing and associated consequences. Many intriguing lead compounds with good biological properties such as antibacterial, antifungal, and anti-inflammatory activity have been identified from marine sources in the hunt for new agents. Similarly, testing of the extracted pigment indicated strong antibacterial action against a variety of harmful bacteria in the current investigation (Shiva Krishna *et al.*, 2019; Shanthi Kumari *et al.*, 2020).

Pathogens resistant to antibiotic pose a threat to the treatment to a wide range of infections. Replacement of the existing antibiotic periodically necessary to prevent the exponential emergence of resistant pathogens (Llic *et al.*, 2005). Development of novel drugs against drug resistant pathogen is the need of the hour. Many fungi are already known to produce antibiotics (Jensen *et al*., 2003). The intensity of inhibition produced by *Thermomyces* sp. was higher when compared to other pigments. Pinkish red pigment from *Sclerotinia* sp. was capable of inhibiting many pathogens except *E. coli* (Perumal *et al.*, 2009). Velmurugan *et al.* (2009) confirmed antibacterial nature of fungal pigments against *S. aureus, E. coli, S. typhi* and *P. aeruginosa*.

Toxicity studies in animal model for the pigment on *Thermomyces* sp. revealed that it did not induce acute or sub-acute toxic effects in mice when adminstraded as oral dose and sub-acute dermal toxicity. There is no noticeable ill effects on growth, organ weight, and histology and hematological parameters even at 2% level (Poorniammal *et al.*, 2011, 2018, 2019a).

Wound healing is a complex and dynamic process by which cellular structures and tissue layers restores itself to its original state. Wound contraction begins a week after wounding at the fibroblastic stage (Vedula *et al.*, 2013; Hu *et al.*, 2017).The present study revealed that topical administration of *Thermomyces* and *P. purpurogenum* pigments at both strengths (1% and 2%) was capable of faster wound healing activity. The wound healing property of the bioactive compounds from *Micrococcus* sp. OUS9 isolated from marine source, resulting the wound healing potential and antimicrobial activity (Shanthi Kumari *et al.*, 2020). This fungal pigment promotes significant wound healing activity by increasing cellular proliferation, formation of granulation tissue, synthesis of collagen and by increase in the rate of wound contraction as compared to control animals. (Al-Henhena *et al.*, 2011; Mohan, 2005).

Wound epithelialization is a process of epithelial regeneration. Post wounding, epithelial cells proliferating and migrate over the wound, thereby providing a protective cover for the freshly formed tissues (Cotran *et al.*, 1994). The result of wound reduction studies indicate that fungal pigment enhanced wound healing in open excision wound. The rate of wound reduction was found to reach a maximum on the 15th day postoperative in treated group. Previous work on the pigments reveals presence of high amount of antioxidants which is the most important components of wound healing (Poorniammal *et al.*, 2019b).

The extract was able to reduce wound bacterial load comparable to the reference drug, ciprofloxacin. At 14 days post treatment, mean total viable count obtained in the fungal pigment treated groups were significantly (p<0.05) lower compared against the untreated control. The absence of irritation and/or pain at wound site in the pigment treated site is an added advantage. Significant increase in the rate of wound contraction and wound re-epithelialization is a reflection of good antibacterial potentials of the pigment both in *in vivo* and *in vitro* antibacterial assay results. In a recent study, Shiva *et al.* (2019) found that red pigment caused a higher reduction in wound area than control. Framycetin ointment treated groups, and that red pigment had considerable wound healing capacity with a beneficial impact on wound repair stages.

5. Conclusion

We confirmed that the fungal pigment from *Thermomyces* sp. had wound healing and antimicrobial activities. The yellow pigment's wound-healing ability is linked to the compound's antibacterial properties. Topical administration of 1% and 2% pigment reduced congestion, edoema, mononuclear leukocyte infiltration, and necrosis, while also increasing epithelialization and granulation tissue development. Our findings suggested that the bioactive pigment might be effective in the treatment of excision wounds or as a wound healing agent in the future.

Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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