Original Research Article

IN VIVO ANTI-MRSA ACTIVITY OF *PENICILLIUM MINIOLUTEUM* ED24

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ABSTRACT

This study was aimed to study the *in vivo* anti-MRSA of *Penicillium minioluteum* ED24, an endophytic fungus residing in medicinal herb *Orthosiphon stamineus* Benth by using rat models. No animal death or local reaction was observed in connection to the application of the test substance during the experimental period. The diameter of wound was concentration-dependent. The higher concentration of the fraction Ma10 used, the smaller wound was observed, where the diameter of wound observed for 2% fraction Ma10 was smaller than 1% fraction Ma10. At the end of the experiment, the lesions on animal models treated with 2% of fraction Ma10 was covered by epithelial tissue and the hair started to grow from the skin. The viable cells obtained from the skin sample were reduced with the increase concentration of fraction Ma10. Besides, the skin sample treated with Ma10 also showed the presence of hair follicles and sebaceous glands relative to the control based on the histological study.

Keywords: Endophyte, MRSA, *in vivo* antimicrobial activity, *Penicillium minioluteum.*

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Running title: *In vivo* anti-MRSA activity of *Penicillium minioluteum* ED24.

INTRODUCTION

Methicilin-resistant *Staphylococcus aureus* (MRSA) can be defined as a strain of *S. aureus* that gained resistance to a large group of beta-lactam antibiotics, which include penicillin and cephalosporin [1]. MRSA is an opportunistic pathogens and it is most common in infecting immune-compromised patients. The worldwide emergence of MRSA is caused by extensive use of antibiotics in the past few decades. Nowadays, MRSA infection is no longer confined to nosocomial infection, but also appears in healthy people in the community with no established risk factor [2]. The recent emergence community acquired MRSA is mainly involving skin and soft tissue infection [3]. MRSA is specially troublesome in hospitals and nursing homes, where patients with weakened immune system are at greater risk on these multi-drug resistant bacterial strains [4]. Topical applications of antibiotics are the most important component of wound infection therapy [5]. Alternative therapies are being sought for treatment of MRSA and one area of interest is the uses of extracts from natural sources.

This study was aimed to evaluate *in vivo* anti-MRSA activity for the dermal administration of dichloromethane extract of *P. minioluteum* ED24, an endophytic fungus previously isolated from medicinal herb, *O. stamineus*. To the best of our knowledge, no scientific evaluation of the therapeutic potentials of this endophytic fungus has been done. Hence this study is very important to evaluate the pharmaceutical potential of this endophytic fungus.

MATERIALS AND METHODS

Endophytic fungus and storage

The endophytic fungus *P. minioluteum* ED24 previously isolated from *O. stamineus* Benth was deposited at Industrial Biotechnology Research Laboratory, Universiti Sains Malaysia, Penang, Malaysia. The fungal isolate was cultivated on Potato Dextrose Agar (PDA; AES) supplemented with powdered host plant materials (5 g/L) and stored at 4 ^{0}C prior to use. The isolate was subcultured on fresh medium every four weeks to ensure purity and viability.

Culture media

Yeast extract sucrose (YES) broth (yeast extract 20 g/L, sucrose 40 g/L, magnesium sulfate 0.5 g/L) supplemented with water extract of *O. stamineus* was used to cultivate the fungus in the shake-flask system. Healthy host plant materials were collected from Balik Pulau, Penang, Malaysia from an area free of fungicides. The plant extract was prepared by boiling 10 g of dried plant material in 500 mL distilled water for 30 min. The extract was filtered and mixed with freshly prepared culture medium and autoclaved at 121 □C for 15 min.

Fermentation and extraction

The inoculum was prepared by introducing two mycelial agar plugs into 250 mL Erlenmeyer flasks containing 100 mL of YES medium. The cultures were grown at 30 ^{0}C in a shaker at 120 rpm. After 20 days of incubation, the fermentative broth and fungal biomass were separated by centrifugation at 5311 g (Sigma; Model 4K15). The freeze-dried fugal biomass was then extracted thrice with dichloromethane (1:50, w/v). The extract was concentrated to dryness using a rotary evaporator under reduced pressure to a crude extract paste.

Fractionation of crude extract

The packing of open column system was performed by using slurry packing method as described by Salituro and Dufresne [6]. Silaca gel 60 with the size of 40-63 μm (Merck) was used as packing material. Twenty five grams of silica gel was used for each glass column with 2.2 cm diameter and 44.0 cm in length. The solvent mixture of hexane and ethyl acetate in the ratio of 10:0 (v/v) was used as mobile phase, followed by increasing polarity with a ratio of hexane and ethyl acetate of 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9 and 0:10 (v/v). Finally, the residual of the extract in the column was eluted by using methanol. The 10th fraction based on the band color, namely Ma10 was collected for the *in vivo* anti-MRSA assay. The fraction collected was red in color.

Preparation of test animals

A total of 40 Sprague Dawley rats (20 male and 20 female) were tested. All the rats were bred and supplied by Animal Research and Service Centre (ARASC), Universiti Sains Malaysia, Penang, Malaysia. All animal protocols used in this study were approved by the Animal Ethics Committee, Universiti Sains Malaysia. Efforts were made to minimize the suffering and number of the rats used.

At the beginning of the study, the rats were 12 weeks old and the body weight was ranged from 200 to 250g. The rats were housed in a group of four and were maintained in opened stainless steel cage (59.5 x 38 x 20 cm^3) with solid bottoms and pine sawdust (Puik, Netherland) was used as bedding. They were kept in a 12 hours light-dark cycle and were cared for in accordance with the regulations for the protection of laboratory animals. The animal cages were sanitized by 70% ethanol daily. Throughout the study period, all the rats were fed a standard rat chow (Specialty Feed, Australia) and tap water was provided without restrictions in polycarbonate bottles. An opaque PVC tube (30 cm long, 12 cm diameter) stuffed with folded paper strips was provided as the nesting materials.

Preparation of extracts for topical use

The fraction Ma10 obtained from the open column chromatography was dissolved in methanol. After that, the fraction was mixed with 100% of petroleum jelly (Vaseline, Malaysia). The cream for topical use was prepared at two different concentrations, 1% and 2% respectively. The mixture was then stored at 4 ^{0}C prior to use.

Inoculation of MRSA on rat models

The ability of the fraction Ma10 to reduce viable bacterial cells was estimated in rats based on previously published studies [7,8]. The rats were divided into five groups (three control and two test groups), with four male rats and 4 female rats in each group. The first control group received no treatment and bacterial inoculation, the second and third group received the vehicle (100% petroleum jelly) and 1% chloramphenicol (drug control) respectively. The two test groups were treated with fraction Ma10 in petroleum jelly at concentration 1 and 2% (w/v) respectively.

The furs of the rats were clipped from about 10% of the body surface area 24 hours before the experiment. The backs of the rats were shaved and swabbed with 70% alcohol. The MRSA infection was established by inoculating 0.1 mL of freshly prepared inoculum on the clipped portion. The bacterial inoculum were injected subcutaneously into the loose skin over the body. The treatment was started at 48 hours after the establishment of the infection by dermal application of 0.05 g Ma10 fraction in petroleum jelly once per day for 5 consecutive days. The body weights of the rats were measured daily during the experimental period. The diameter of the wound was measured using a ruler. At the end of the treatment, animals were euthanized with carbon dioxide and the skin sample was excised at the infection site. The excised skin samples were kept in sterile physiological saline and 10% formaldehyde respectively.

Calculation of colony forming unit of the skin sample

To estimate the viable bacterial cells in the skin sample, 1 g of the skin sample excised from the infection site was dissected and transferred into micro-centrifuge tube with 1 mL of sterile physiological saline. The samples were then centrifuged at 1500 g for 10 minutes. The suspension was serially diluted and aliquots of 50 µL from each of the diluents were plated on Mannitol salt agar (Oxoid) and incubated at 37 ^{0}C for 24 hours. The colony forming units of each the sample was calculated.

Histological study of the skin sample

After the fixation of the sample in 10% formaldehyde for overnight, the sample was dehydrated to remove the fixative and water from the tissue. The sample was dehydrated gradually in a graded ethanol series through 50%-70%-80%-90%-95%-100% ethanol. The sample was then cleared with xylene for 30 minutes. The cleared sample was impregnated with molten paraffin at 60 ^{0}C, with four changes in 7 hours. Then the sample was embedded with molten paraffin wax in a mould on a cold plate. A single-edge razor was used to trim the opposite site of the paraffin wax block after detaching the sample from the mould. Microtome knife was used to section the paraffin block, the thickness of each section was 5 µm. The slides containing

the paraffin sections were dehydrated with Histosolve and 100%-90%-70%-50% ethanol series. Hematoxylin and eosin staining was performed as per described by Teng *et al.* [9]. The slides were then observed under light microscope.

RESULTS AND DISCUSSION

In order to study the topical anti-MRSA effect of fraction Ma10, the *in vivo* rat models were used. No animal death or local reaction was observed in connection to the application of the test substance during the experimental period. On the second day after the inoculation of bacterial inoculums, the infected area appeared as a red and swollen bump. The formation of white-yellow colored pus was observed on the infection site.

Figure 1 shows the body weight of the rat models during the 7 days of experimental period. From day 1 to day 3, no significant difference was detected on the body weight of the rat models for all the test and control groups (p □ 0.05). However, at day 7, the body weight of the rat models received placebo control showed significant reduction in their weight gain compared to the drug control and test group (p □ 0.05). The deterioration of weight gain can be due to the MRSA infection that reduced the food consumption of the rat models as the symptom of itchiness may reduce the appetite [6, 10].

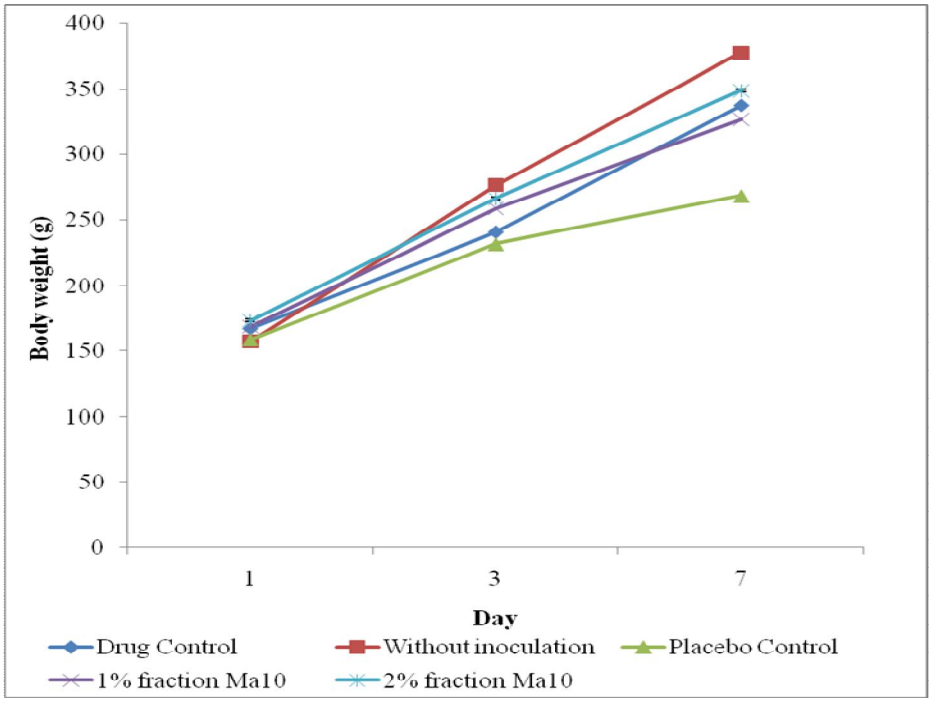


Figure 1: The body weight of the MRSA-infected rat models received different treatments. At day 7, the body weight of the rat models received placebo control showed significant reduction in their weight gain compared to the drug control and test group.

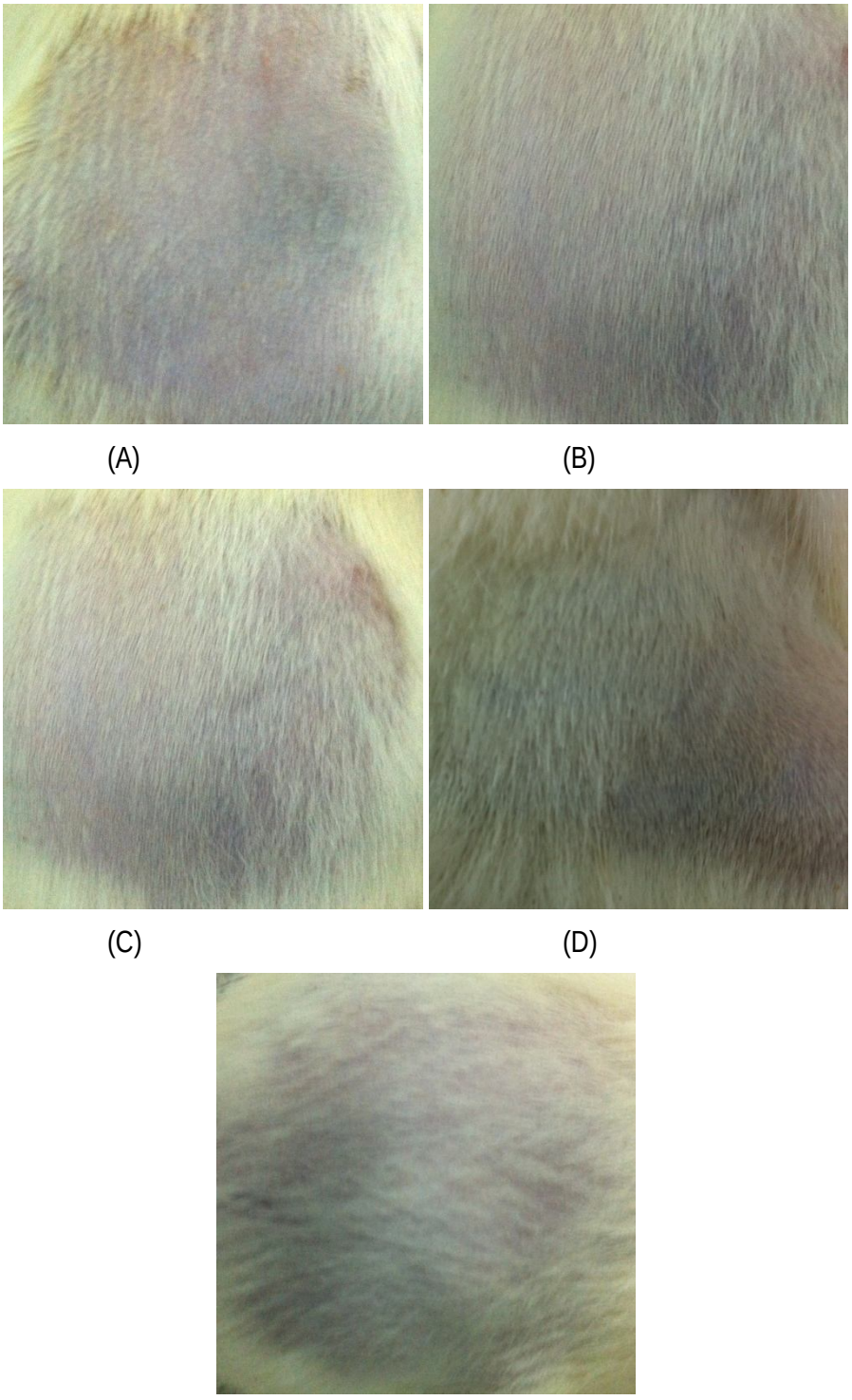
On day 1, no wound formation was observed for all the test and control groups (Figure 2 A-E). However, on day 3, the formation of wound was observed, but no significant difference was observed in the wound diameter for all the test and control groups (Figure 3 A-E). The topical application of fraction Ma10 reduced the diameter of the wound relative to placebo control. On day 7, the pus formation was still observed on the rats receiving placebo control, and the symptom of inflammation was obvious on the infection site (Figure 4A). The wounds received the drug control and 2% fraction Ma10 were fully recovered (Figure 4 B,D). The lesion was covered by epithelial tissue and the hair started to grow from the skin. Besides, the redness symptoms observed on the infection site applied with 1% (Figure 4 C) or 2% of fraction Ma10 was reduced. The anti-inflammatory of the fraction was due to the presence of 2,6-dimethoxy-*p*-benzoquinone as Teng and Lim [11] reported the anti-inflammatory activity of this compound. The diameter of wound was concentration-dependent. The higher concentration of the fraction Ma10 used, the smaller wound was observed, where the diameter of wound observed for 2% fraction Ma10 was smaller than 1% fraction Ma10. Both of these symptoms were not observed on the rats treated with 1% fraction Ma10, indicating the growth of MRSA was inhibited. Besides, it is also note that the rats that received no MRSA inoculation exhibited no symptom of MRSA-infection during the experimental period (Figure 4 E). Table 1 summarizes the diameter of wound on the infection site for different treatments.

Table 1: The diameter of the wound of MRSA-infected rat models received different treatments. On day 7, the wounds received the drug control and 2% fraction Ma10 were fully recovered.

Treatment	Diameter of wound (mm)		
	Day 1	Day 3	Day 7
Drug control	-	7.6 ± 0.5	-
Placebo control	-	6.3 ± 1.4	27.3 ± 1.1
Without inoculation	-	-	-
1% fraction Ma10	-	8.1 ± 1.4	14.7 ± 0.7
2% fraction Ma10	-	6.9 ± 0.8	-

- No wound was observed

The viable cell count was conducted to enumerate the MRSA cells present in 1 g of skin sample excised from the infection site. The results are presented in Table 2. Only the colony that closely resembles the colony morphology of MRSA was calculated as one colony forming unit. The topical application of fraction Ma10 significantly reduced the bacterial count in the rat models ($p < 0.05$). The anti-MRSA effect of fraction Ma10 was concentration dependent. The viable cells obtained from 1g of skin sample were reduced with the increase concentration of fraction Ma10. One percent of fraction Ma10 exhibited bacteriostatic effect on MRSA at the infection site, and it also reduced the symptoms of MRSA infection. Two percent of fraction Ma10 exhibited bactericidal effect on MRSA, where more than 99.9% of bacterial cells were killed in relative to the control.



(A) (B)

(C) (D)

(E)

Figure 2: The MRSA-infected site on the skin of the rat models on day-1 for (A) Placebo, (B) Drug control, (C) 1% fraction Ma10 treatment, (D) 2% fraction Ma10 treatment and (E) Without MRSA inoculation.

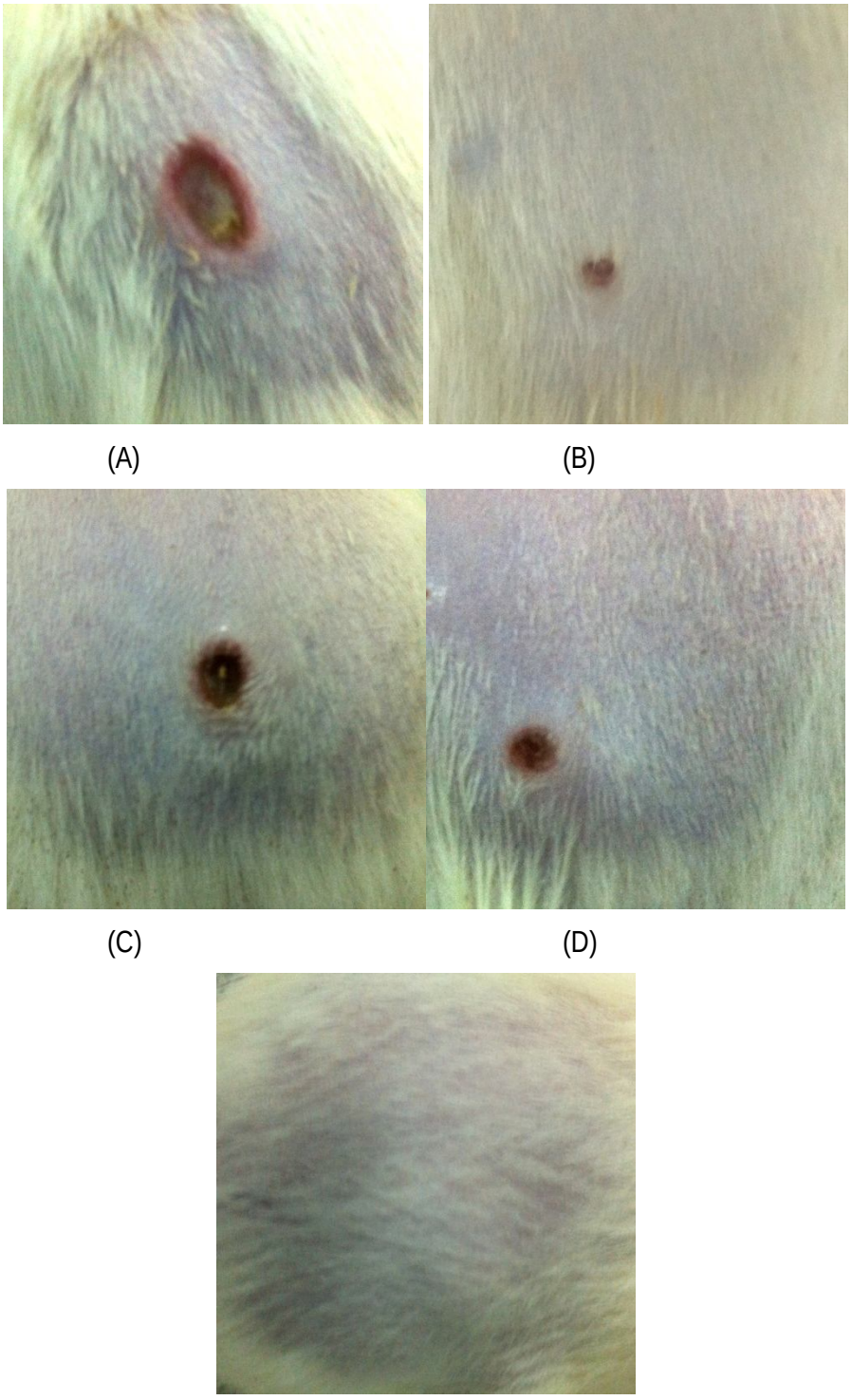
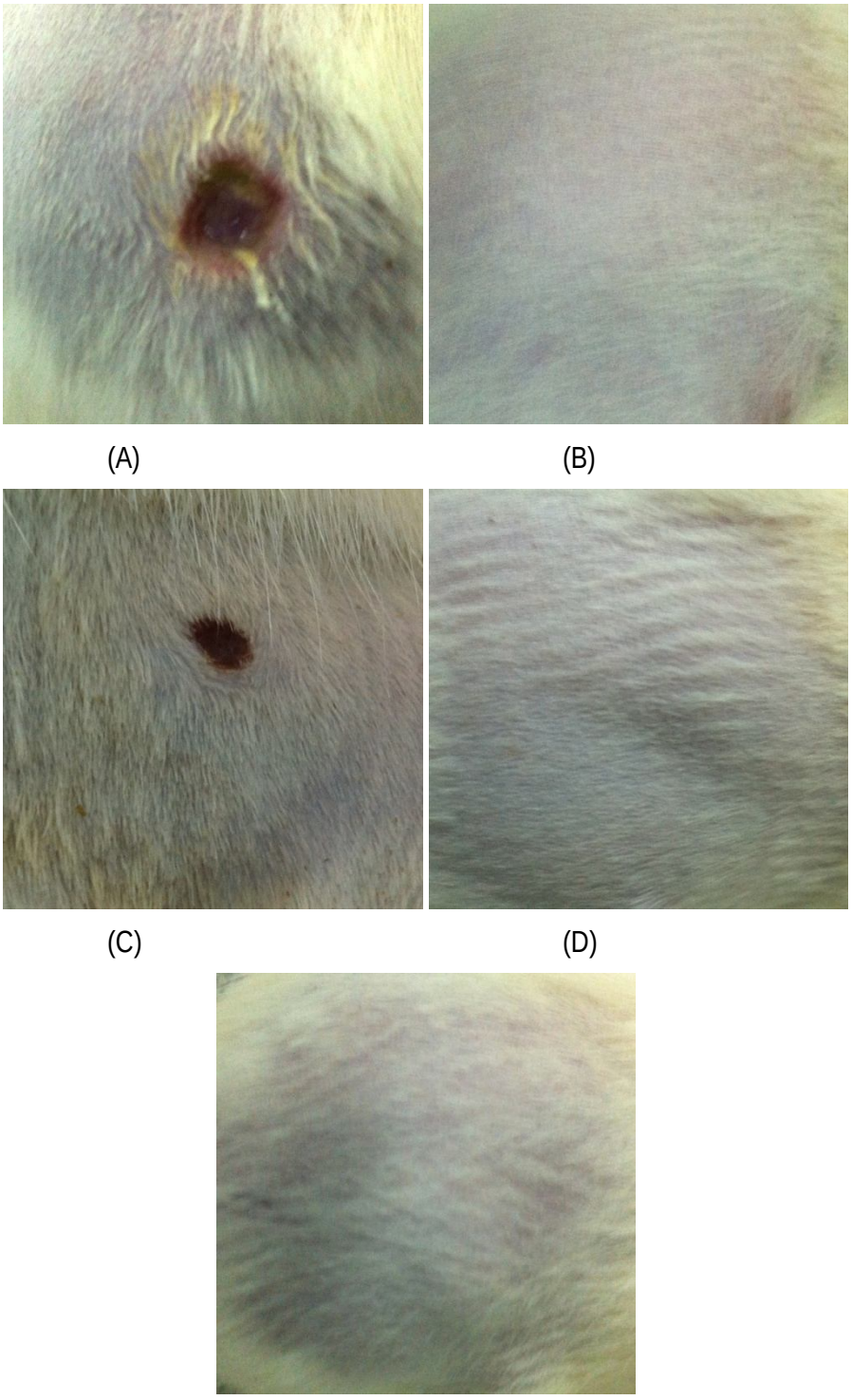


Figure 3: The MRSA-infected site on the skin of the rat models on day-3 for (A) Placebo, (B) Drug control, (C) 1% fraction Ma10 treatment, (D) 2% fraction Ma10 treatment and (E) Without MRSA inoculation.



(A) (B)

(C) (D)

(E)

Figure 4: The MRSA-infected site on the skin of the rat models on day-7 for (A) Placebo, (B) Drug control, (C) 1% fraction Ma10 treatment, (D) 2% fraction Ma10 treatment and (E) Without MRSA inoculation.

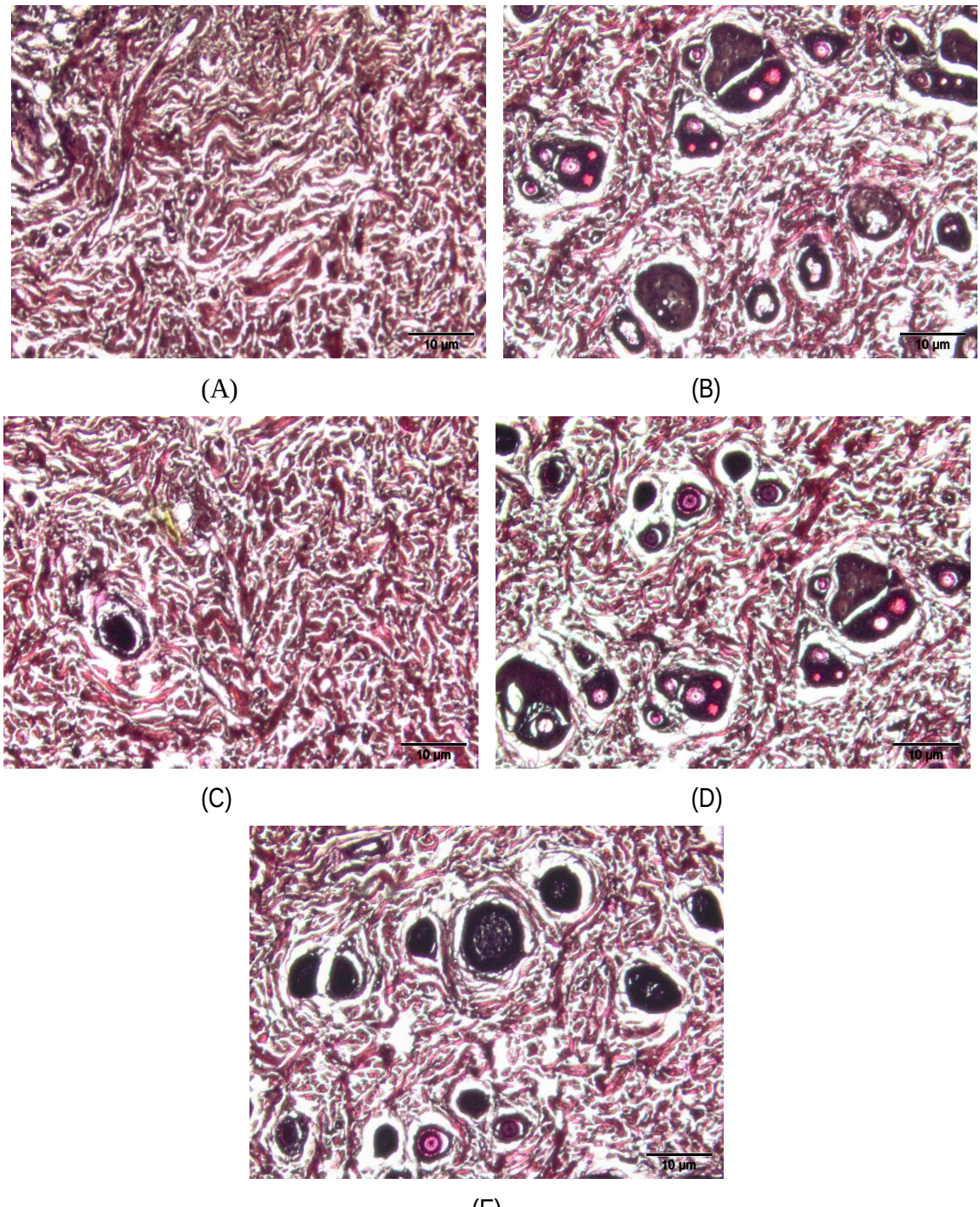


Figure 5: The histological analysis of the skin sample of MRSA-infected rat models that received (A) Placebo control, (B) Drug control, (C) 1% fraction Ma10, (C) 2% fraction Ma10 and (E) Without MRSA inoculation.

Table 2: The viable cell count of MRSA cells obtained from 1 g of skin sample of MRSA-infected rat models. The topical application of fraction Ma10 significantly reduced the bacterial count of MRSA.

Treatment	Viable cells count (CFU/g)
Drug control	98
Placebo control	8.7×10^9
Without inoculation	-
1% Ma10 fraction	5.6×10^5
2% Ma10 fraction	1.3×10^2

Figure 5 shows the histological analysis of the skin sample from the rat models urderwent different treatments. The histological analysis of the skin sample without MRSA inoculation (Figure 5 E) showed the presence of healthy hair follicles and sebaceous glands in the dermis layer. In contrary, the histological analysis of the placebo control group (Figure 5 A) showed the structure of damaged skin, with no hair follicle and sebaceous glands. The findings were in agreement with Nichols and Florman [5, 12] where the hair follicle and sebaceous gland of the skin tissue were ruptured after infected with MRSA. For the rat models treated with 1% fraction Ma10 (Figure 5 C), the regeneration of hair follicle was observed in the dermis layer, marking the improvement in the wound healing. For the drug control and 2% fraction Ma10 test group (Figure 5 D), the histological studies of the skin were similar to the sample without inoculation, with widespread of hair follicles and sebaceous glands in the dermis layer of the skin. The results showed that 2% of fraction Ma10 significantly improve the regeneration of hair follicles and sebaceous glands of the skin, by inhibiting the growth of MRSA.

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