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AQUEOUS EXTRACT EMULSIFYING OINTMENT OF *Marphysa moribidii* (ANNELIDA: POLYCHAETA) INCREASES COLLAGEN DEPOSITION IN WOUND HEALING MODEL AT LOW CONCENTRATION

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ABSTRACT

Acute wound cases are increasing every year. Meanwhile, current treatments have many adverse impacts; thus, alternative treatment is required. *Marphysa moribidii*; a local polychaete is found to has a promising potential as a wound-healing agent due to its regenerative capability. However, no prior study has been conducted to prove this notion. Hence, this study is aimed to determine the effectiveness of aqueous extract of *M. moribidii* in wound healing treatment. The polychaete was finely pulverized and lyophilized by freeze-dryer to form a powdery-form extract before preparing in three different concentrations: 0.3% (w/w), 1.0% (w/w), and 2.0% (w/w) in ointment form. The treatments including Gamat oil 0.4% (w/w) as control were applied to the rat model once daily for 14 days. Gamat oil (0.4%) demonstrated the most rapid wound healing, followed by polychaete ointment (0.3%). However, based on Masson's trichrome staining, the polychaete ointment exhibited the most collagen deposition compared to other treatments. The staining indicates a more effective healing process of the wound after treated with the polychaete ointment. Based on the findings, polychaete extract has great potential in wound healing; more detailed studies are needed to gain more evidences.

Key words: Acute wound, collagen formation, *Marphysa moribidii*, polychaete, wound healing

INTRODUCTION

Pathologically, a wound refers to multiple types of injury that usually form on the outermost layer of an organism; the epidermis of skin (Takeo *et al*., 2015; Chhabra *et al*., 2016). It can be divided into two categories which are acute and chronic wounds. The acute wound heals within a predictable time frame while chronic wound usually takes a more extended period. Both wounds undergo several actions of normal wound healing process such as inflammatory response, granulation tissue formation, which includes reepithelization and angiogenesis, and matrix remodeling (Schultz *et al*., 2011). Nowadays, the wound has been a challenging clinical problem (Kayode *et al*., 2017). Particularly when an acute wound has progressed into a chronic wound. More severe complications including inflammation, infection, or constant pressure will occur. Subsequently, it may lead to limb amputation among diabetes patients (Han & Ceilley, 2017).

Considering various limitations with current treatment, wound healing treatment from natural resources such as invertebrates appears as one alternative. Invertebrates have been known for their potential in wound healing or medical applications.

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For instance, earthworms *Lumbricus terrestris* can fasten the wound healing process due to its regenerative properties (Cikutovic *et al*., 1999). However, the interference of chloride compounds in membranes of macrophage inhibited cellular function and suppressed the healing capability of the earthworms (Goven *et al*., 1993; Ville *et al*., 1995; Giggleman *et al*., 1998). Also, sea cucumber (gamat) species, *Holothuria glaberrima*, is another example of invertebrates in wound healing. It was revealed that gamat's wound healing properties correspond to those that occurred during the organism organ regeneration (Pangestuti & Arifin, 2018). However, the species is facing extinction issues and may overshadow its medicinal applications (Hoek *et al*., 2017; Rahman & Yusoff *et al.*, 2017). Therefore, polychaetes can be an alternative for wound healing treatment due to their high species abundance and diversity. However, less study has been explored on polychaetes, especially in Malaysia.

Polychaete is another type of invertebrate and has vast potential in wound healing treatment. It is also known as bristle worms belong to the class Polychaeta, the most diverse class in Phylum Annelida. They have an elongated and segmented body plan. Currently, about 13,000 polychaete species have been described worldwide, and more remain to be discovered (Read & Fauchald, 2020). In Malaysia, only 64 polychaete species from 31 families have been identified (Idris & Arshad, 2013; Idris *et al.*, 2014). The polychaete species used in this study is *Marphysa moribidii* or locally known as 'Ruat Bakau'. The species is one of the local polychaetes that live along the west coast of Peninsular Malaysia harvested as bait worms (Idris & Arshad, 2013). Based on the observation in the field and the laboratory, *M. moribidii* has the ability for posterior regeneration, which indicates the possibility of the species as a wound-healing agent. However, there is no prior study on the involvement of *M. moribidii* in wound healing. Hence, this study was conducted to determine the efficacy of local polychaete (*M. moribidii*) extracts in wound healing via *in vivo* study.

MATERIALS AND METHODS

Samples collection

M. moribidii samples were collected from the upper tidal flat zone in the mangrove area next to Pantai Morib, Selangor, Malaysia during the low tide. The soil at the mangrove area near *Rhizophora* sp. was dug and the polychaetes were removed gently from the soil. Then, the polychaete samples were kept at -20°C before the experiment.

Preparation of polychaete aqueous extract

Approximately 20 g of polychaete samples were weighed. Samples were pulverized using pestle and mortar. The samples were homogenized without water. After that, the distilled water was added to the samples and soaked overnight. The filtered water was then collected in a flask (flask A). The polychaetes tissue was soaked again for 4 hr and followed by centrifugation at 3000 rpm for 20 min. The resulted supernatant was collected in another flask (flask B). Both liquids in flask A and B were mixed and stored at -20°C. After 24 hr, the samples were lyophilized by freeze dryer to produce a powdery-like extract. Freeze-dried samples were used in this study because dried samples do not undergo further biochemical reactions and have fewer chances of contamination. Then, the extract of *M. moribidii* was prepared in ointment form in different doses i.e. 0.3% (w/w,), 1.0% (w/w), and 2.0% (w/w) by mixing with cetamacrogol emulsifying ointment (paraffin ointment), based on a study by Mazlidiyana *et al*. (2017). The mixture was mixed well until homogenous and ready to apply to the wound.

Animal husbandry

In this study, Sprague Dawley female rats (n=24) were used. The rats were monitored every two days and kept in a standard condition with free access (*ad libitum*) to food (commercial pellet) and water at the Institute Marine Biotechnology, Universiti Malaysia Terengganu (UMT). Each rat was kept in an individual cage to minimize the chances of infection and reduce stress.

The experiment was conducted by dividing the treatment into six groups (four rats per group) based on the ointment concentrations, including positive and negative controls. For the negative control group, no wound healing treatment (T1) was used. Commercial products containing acriflavine (T5) and gamat oil (T6) were used as positive controls. Three different concentration of polychaete extracts i.e., 0.3% (w/w) (T2), 1.0% (w/w) (T3) and 2.0% (w/w) (T4) polychaete aqueous extract was applied to wound once a day for two weeks. The animal study was performed in the Institute of Marine Biotechnology, UMT. The procedures were reviewed and approved by the UMT Animal Ethics Committee (Registration no: UMT/JKEPHT/2017/10).

Wound healing model

The wound healing model used in this study is the excisional acute wound model, which is based on Dunn *et al*. (2013) and Park *et al*. (2014) with little modifications. A full-thickness circular wound, with a diameter of 8mm were created on both back

sides of a rat using a biopsy punch. The procedure was conducted after induction of deep anesthesia with intramuscular injection (1 mL/kg) of a mixture containing ketamine 500 mg, xylazine 20 mg, and 8.5 mL of 0.9% saline or PBS. The fur around the neck to further down back and both flanks was removed with a hair clipper or razor.

Then, the topical application of treatment was carried out, according to Tsai *et al.* (2014). The size of the wound area recorded for the wound contraction process was measured immediately by placing a transparent polythene graph paper over the wound and then tracing the area of the wound on it. Then, rats were sacrificed after it was completely euthanized by showing no tickle on its whiskers and no pain reaction when pressed on its leg palms. The rats were sacrificed at day 7 (n=12) and 14 (n=12) for histological evaluation.

Wound contraction

The morphological changes in wound contraction of the rats were monitored for 14 days throughout the study. It was recorded every two days after the wounding operation.

The percentage of wound closure was calculated as:

$$\%\text{Wound closure} = \frac{\text{(Wound area of day 0 – Area of the inner circumference on day X)}}{\text{(Wound area of day 0)}} \times 100$$

where X= 0, 2, 4, 7, 10 and 14 days.

Histology analysis

Skin samples (approximately 1 × 1cm²) with the wounded area were dissected from sacrificed rats for all treatments on day 7 and 14. For histological works, the skin tissue was fixed in 10% neutral buffered formalin (NBF) for 24 hr. Then, the tissues were dehydrated with 70%, 80%, 95%, and 100% ethanol and paraffin infiltrated by a tissue processor machine for 24 hr. Then, the tissues were embedded in a solid paraffin block for the preparation of tissue ribbon. The prepared slides were divided into two groups before staining. The first group was stained using hematoxylin and eosin (H&E) staining for general histological observation. The second group was stained using Masson's trichrome (MT) staining to observe the collagen deposition and its arrangement in the wounded tissue. All stained slides were observed under light microscopy (Suvik & Effendy, 2012).

Statistical analysis

The wound contraction data between each treatment were analyzed and compared using One-way ANOVA and posthoc test (Mazlidiyana *et al*., 2017). The test was chosen to determine whether polychaete extracts can heal the wound significantly than commercial ointment treatment. The significance level used in this study is 5% (p=0.05).

RESULTS AND DISCUSSION

Wound healing process

Approximately 1.5–1.7 g of *M. moribidii* crude extract in a powdery-form was produced from 20 g of the polychaete after the freeze-drying procedure. It was enough to prepare three concentrations of polychaete ointment as treatment in this study, i.e. 0.3% (w/w), 1.0% (w/w) and 2.0% (w/w). After applied the polychaete ointment daily for 14 days, wounded rats from group T2, which was treated with 0.3% (w/w) polychaete extract showed the most visible results with less scar observed (Figure 1). The result was supported by wound contraction data displays in Figure 2, in which the same concentration showed the second-highest wound contraction (96.91%) after completed the treatment for 14 days. Intriguingly, the treatment displayed the fastest wound contraction within the first four days compared to other treatments. However, in general, the visual observation (Figure 1) and wound contraction (Table 1) data demonstrated gamat oil (0.4%) showed the most competitive findings with less scar observed and the highest wound contraction (99.17%) after completed the treatment period.

Meanwhile, polychaete ointment treatment at 1.0% (w/w) showed similar results with acriflavine treatment at concentration 0.4% (w/w). Interestingly, the highest concentration of polychaete crude extract (2.0% (w/w)) showed no difference when compared to the negative control (wound model without any treatment). The results were not as expected, which low concentration of polychaete crude extract displayed more effective results than the higher concentration. It is probably the presence of bioactive compounds which are only can be activated in the wound healing process in a small quantity only and yet to be investigated in the future.

Wound healing activity recorded after the application of polychaete ointment on rats in group T2 as expected. *Marphysa moribidii* is a polychaete with self-generative capability. The effects should also be observed after apply it as a wound healing treatment. According to Zoran & Martinez (2009), it is reported that polychaete species, *Eurythoe complanata* enhanced the wound healing process due to its ability to regenerate body segments. In another study, *Enchytraeus japonensis* was used as a model to study the expression of genes during regeneration. It is stated that known genes play vital roles in protein function and as

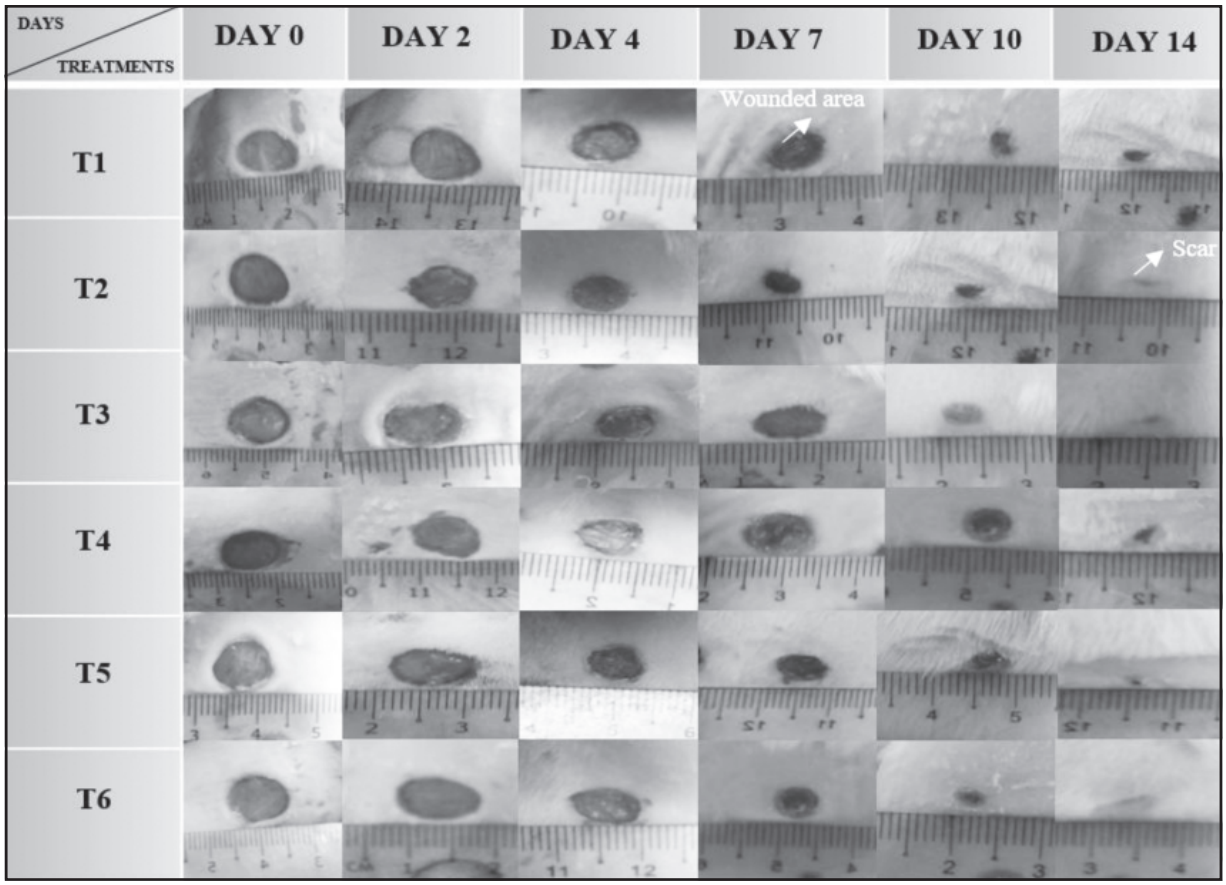

Fig. 1. Visual observation of wound healing process and wound size between different groups of treatment. T1=Negative control (no treatment), T2=0.3% (w/w) polychaete extract treatments, T3=1.0% (w/w) polychaete extract treatments, T4=2.0% (w/w) polychaete extract treatments, T5=0.4% (w/w) acriflavine (positive control), T6=0.4% (w/w) gamat oil (positive control).

wells as in regeneration (Myohara *et al*., 2006). However, the exact bioactive compounds involved in the wound healing process is still unknown. Meanwhile, the effectiveness of gamat oil in wound healing is well-known. For example, gamat or sea cucumber species, *Stichopus horrens* was reported to exhibit wound healing properties due to its fatty acid composition in the extract (Fredalina *et al*., 1999). However, extinction issue related to the species is worth to be considered to avoid over-harvesting and over-exploitation of gamat as a wound-healing agent. Subsequently, it can be replaced with the polychaete species, which are abundantly and less explored, especially in Malaysia.

Histopathological analysis

Histopathological analysis of the wound healing process is related to cutaneous wound repairing phases which have been divided into three phases. The phases are inflammation, proliferation, and epithelization, which determine the structure and strength of healed tissue (Mantle *et al*., 2001). To understand the phases in detail, histological analysis on the wounds using H&E staining was performed on day 7 and day 14 after the treatments. H&E stain was used to observe the morphological changes in the wounded area of rat's skin such as the formation of blood vessels, the formation of the hair follicle, fibrins, fibroblasts, granulation tissue between the wounded part at different groups of treatments on day 7 and 14. Hematoxylin is a deep purple color, and it stains nucleic acid, whereas Eosin is pink stains proteins.

Results from the H&E staining on day 7 showed all treatments stimulate the formation of blood vessels, which indicates the inflammation stage in the wound healing process (Figure 2(i)). The vasoconstriction of blood vessels and platelet aggregations during this stage limits further damage to the wound by preventing the flow of blood. Results revealed that scabs were formed for day 7 treatment since the blood became dry. Inflammatory cells contribute to the release of cytokines which remove cell debris and promote angiogenesis (Wallace & Zito *et al.*, 2019). Based on the H&E staining analysis on day 14, all groups of treatment and positive controls show the high formation of granulation tissue compared to the negative control (without treatment). The scab was no longer visible,

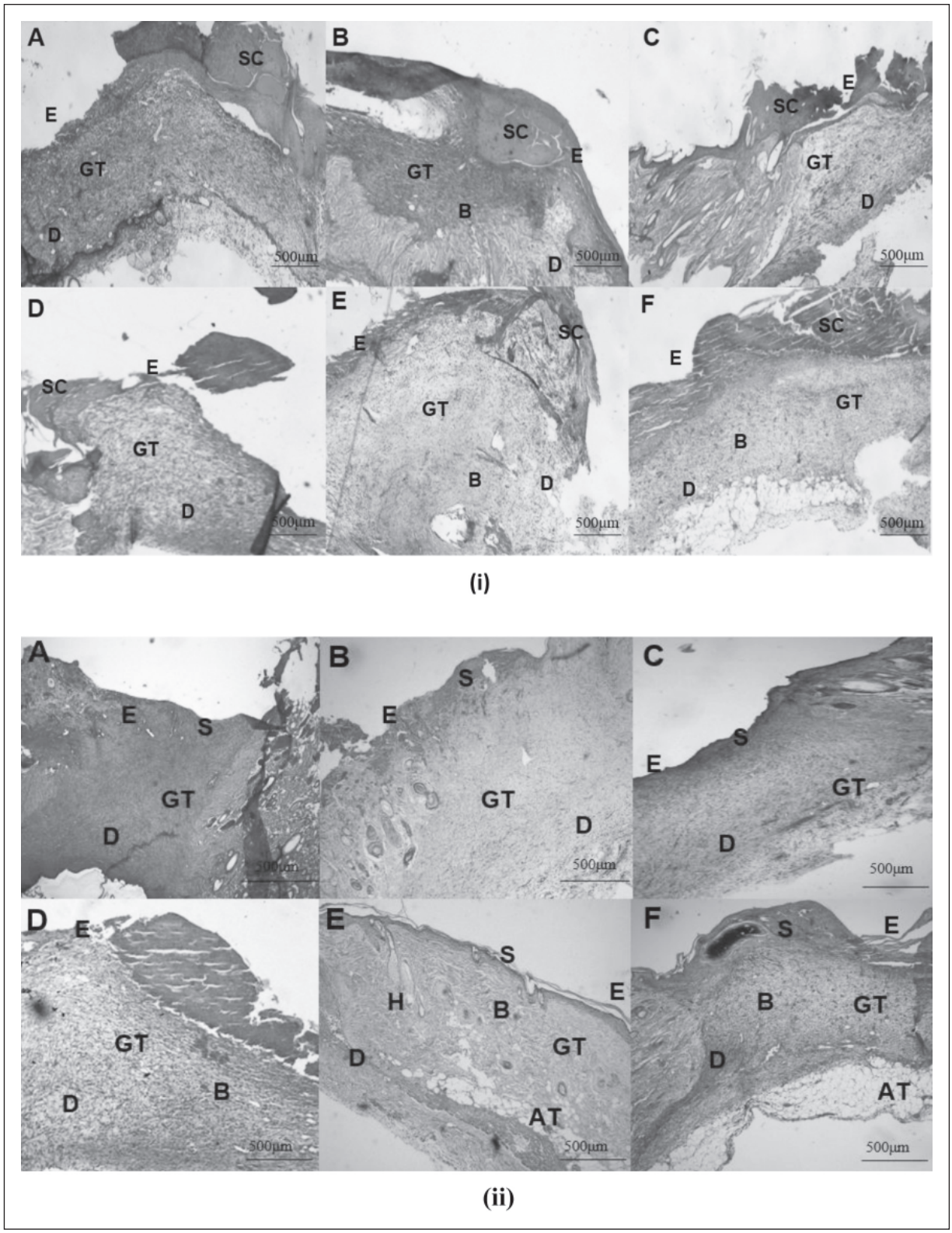

Fig. 2. Photomicrograph of histopathological analysis of wounds treated with different groups of treatments on rat's skin. (i) day 7 and (ii) day 14. A: Negative control (no treatment), B: 0.3% polychaete extract treatments, C: 1.0% polychaete extract treatments, D: 2.0% polychaete extract treatments, E: 0.4% (w/w) acriflavine (positive control), F: 0.4% (w/w) gamat oil (positive control). Note: AT: Adipose Tissue; B= Blood Vessel; D= Dermis; E= Epidermis; GT= Granulation tissue; S= Scab. n=4. Sildes were stained with hematoxylin and eosin staining. Magnification at 50X.

Table 1. The rate of wound contraction of different groups of treatments with measurements taken from the day of wounding (day 2) until day 14

Post wounding days	Treatments					
	T1	T2	T3	T4	T5	T6
	Wound area (mm^2) and percentage of wound contraction					
Day 2	67.13±2.18 (10.80%)	55.00±1.17 (26.25%)	60.25±5.23 (19.93%)	62.13±6.66 (17.44%)	56.50±3.26 (24.92%)	58.63±6.33 (22.09%)
Day 4	52.18±3.05 (30.66%)	40.73±2.17 (45.88%)	51.28±5.25 (31.86%)	50.85±5.36 (32.43%)	42.93±3.5 (42.96%)	47.93±5.07 (36.31%)
Day 7	39.70±1.58 (47.24%)	27.25±5.09 (63.79%)	29.65±5.04 (60.60%)	33.88±7.68 (54.98%)	29.50±2.47 (60.80%)	23.38±5.71* (68.94%)
Day 10	13.48±0.94 (82.09%)	6.80±1.37* (90.96%)	11.55±1.33 (84.65%)	12.43±1.44 (83.49%)	8.95±1.29* (88.11%)	5.23±1.58* (93.06%)
Day 14	4.58±0.54 (93.92%)	2.33±0.22* (96.91%)	2.98±0.43* (96.05%)	4.28±0.83 (94.32%)	2.40±0.65* (96.82%)	0.63±0.24* (99.17%)

Note: T1=Negative control (no treatment), T2=0.3% (w/w) polychaete extract treatments, T3=1.0% (w/w) polychaete extract treatments, T4=2.0% (w/w) polychaete extract treatments, T5=0.4% (w/w) acriflavine (positive control), T6=0.4% (w/w) gamat oil (positive control). Data are presented as mean values ± with standard deviation, (n=4). The value of $p<0.05$ was considered to be statistically different, symbol (*). The analysis was performed using one-way ANOVA analysis.

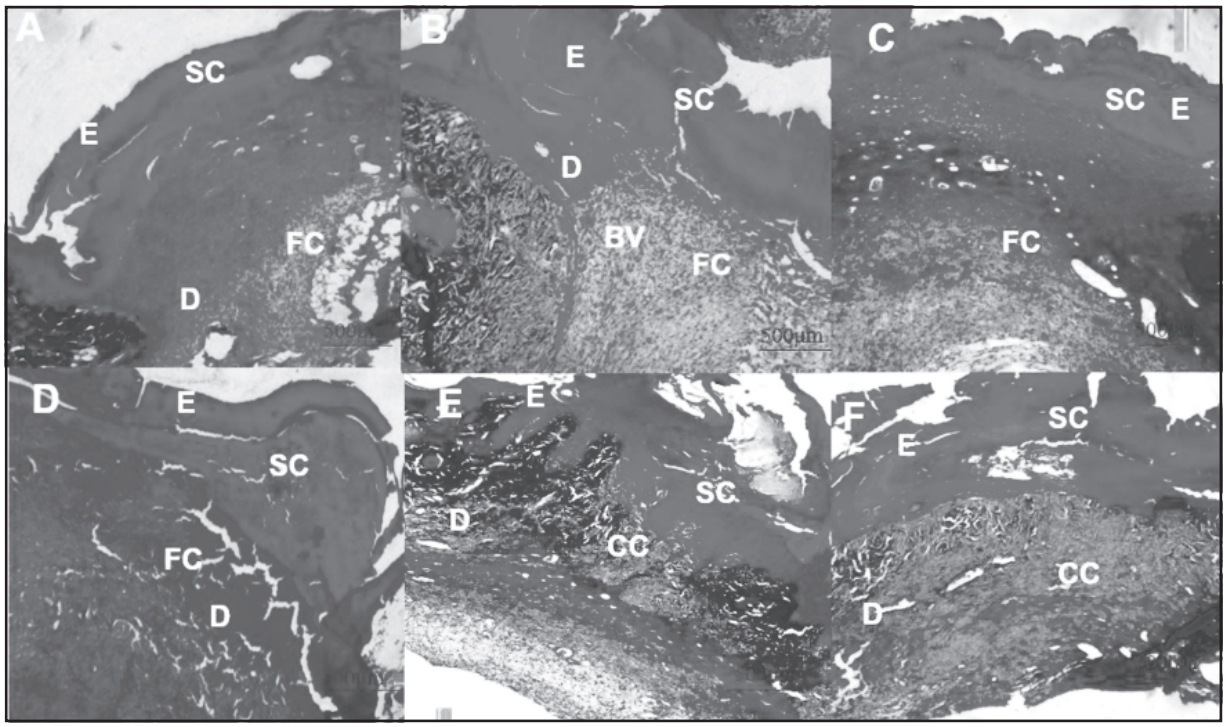

Fig. 3. Photomicrograph of histopathological analysis of wounds treated with different groups of treatments on rat's skin. A: Negative control (no treatment), B: 0.3% (w/w) polychaete extract treatments, C: 1.0% (w/w) polychaete extract treatments, D: 2.0% (w/w) polychaete extract treatments, E: 0.4% (w/w) acriflavine (positive control), F: 0.4% (w/w) gamat oil (positive control). 0.3% polychaete extract shows more collagen deposition compared to normal (without treatment). It also shows rapid wound healing based on collagen deposition which can be comparable to commercialized products, gamat oil, and acriflavine even applied at a lower concentration. Note: BV= Blood vessel; CC = Coarse Collagen Fibre; D= Dermis; E= Epidermis; FC= Fine Collagen Fibre; F= Fibroblast; S= Scab; n=4. Slides were stained with Modified Masson's trichrome staining. Magnification at 50x. These images were taken from rats on day 7.

and the wound contraction became higher on day 14. The granulation tissue, which consists of new blood vessels, fibroblasts and myofibroblast indicate proliferative stage in wound healing. According to Chen *et al*. (2011), myofibroblast promotes wound healing as it will grip the wound edges and undergo contraction using a mechanism similar to that in smooth muscle cells. Fibroblasts break down the fibrin clot to create a new extracellular matrix (ECM) and collagen structures to support the other cells associated with effective wound healing (Bainbridge *et al.*, 2013). The microscopic observation of (Figure 2(ii)) showed that the growth of hair follicles is continuous with the interfollicular epidermis in group T2, T5, and T6 compared to other groups of treatment. The results may indicate the treatment demonstrated the effective effects on the wound. Jahoda and Reynolds (2001) reported that further works need to be done to understand the relationship between the hair follicle and epidermis in wound healing.

Remodeling is the last stage of the wound healing process as it takes place by further covalent cross-linking of collagen molecules. Although H&E staining is well established, the stain unable to differentiate important histopathological analysis such as collagen deposition (Ukong *et al*., 2008). In this study, modified Masson's trichrome staining (MT) was used to evaluate fibroblast proliferation, collagen formation, and re-epithelization, and wound healing processes (Suvik & Effendy *et al.*, 2018). Based on MT stain (Figure 3) at day 7, the collagen deposition in blue color in group T2 is higher compared to other groups of treatments. The results demonstrated clear differentiation between collagen fibers (blue staining) and normal muscle (bright red staining). High deposition of collagen in T2 indicates lower intensity and width of the wound area, which indicates a successful heal of a wound. In contrast, positive control; gamat oil, displayed thin epithelial layer and thick collagen bundles with lesser blood vessel formation on day 7 treatment. Meanwhile, negative control (without treatment) showed a lower amount of collagen deposition and synthesis compared to other groups of treatment. Aqueous extract *M. moribidii* emulsifying ointment at the concentration of 0.3%, acriflavine, and gamat at 0.4% concentration exhibited lower inflammatory cells with collagen fibers in the wounded area compared to other groups. The newly formed tissues gained tensile strength and flexibility, and ease the wound healing process. According to Myllyharju and Kivirikko (2001), collagen plays a vital role in maintaining the structural integrity and healing wound. The structural and molecular interactions have been reviewed since collagen can be a material choice for wound healing and tissue applications (Chattopadhyay & Raines *et al.*, 2014). MT staining on day 14 cannot be done because of a technical issue, i.e. the tissue crumbles during tissue sectioning. The problem may due to over-processing where the tissue has been incompletely dehydrated.

CONCLUSION

This research was carried out to determine the efficacy of wound healing treatment using polychaete aqueous extract, *M. moribidii* in ointment form. The results reported that 0.3% (w/w) polychaete crude extract showed the optimal concentration for wound healing contraction using the rat model. Microscopic observation using H&E and MT staining further proved that the polychaete extract can heal the wound and stimulate collagen formation at the lower concentration (0.3% (w/w)) compared to the higher concentrations (1.0% (w/w) and 2.0% (w/w)). Moreover, the effectiveness of wound healing treatment using polychaete extract at low concentrations is comparable to the established current treatment (antiseptic; acriflavine, and gamat oil). Further studies should be carried out to know the exact bioactive compounds involved as wound healing agents.

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