UNPAD

Indonesian Journal of Pharmaceutical Science and Technology
Journal Homepage : http://jurnal.unpad.ac.id/ijpst/

Application of Common Greenbottle Fly (*Lucilia sericata* Meigen, 1826) Larvae Extract for Incision Wound Treatments in Rats

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Submitted 8 October 2018; Revised 13 December 2018; Accepted 11 January 2019; Published 31 March 2019
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Abstract

Common green-bottle fly (*Lucilia sericata*) larvae have been used in maggot debridement therapy to promote wound healing since the 19th century following the emergence of an antibiotic-resistant strain of bacteria. Whole body extracts and hemolymph of *L. sericata* larvae shown antibacterial properties. This research aims to examine the ethanol extract from whole body of *L. sericata* larvae to accelerate the wound healing on the skin of male Wistar rats (*Rattus norvegicus*). The method was a completely randomized design with six treatments and four replications each. The incision wound created at the dorsolateral region of shaven skin at ±1.5 cm using sterile scissors. The extract at concentration 5, 10 and 20% in olive oil were applied topically to wounded rats, as well as Betadine® for the reference group. At day 15th, the wounded site harvested, fixed in 10% NBF, embedded in paraffin, and sectioned at 5-7 µm, then stained with hematoxylin-eosin or trichrome Heidenhain's Azan for histological examination. The results showed that topical application of the *L. sericata* larvae extract at concentration 10% was significantly recover the wounded skin by enhanced re-epithelialization, narrowed granulation tissue, as well as increased capillary number and collagen density than other treatments ($p<0.05$). Overall, our data support the *L. sericata* larva extract as an agent to accelerate the wound healing process on skin.

Keywords: ethanol extract, green-bottle fly, incision wound, whole body larva, wound healing.

Aplikasi Ekstrak Larva Lalat Hijau (*Lucilia sericata* Meigen, 1826) untuk Obat Luka Insisi pada Tikus

Abstrak

Larva lalat hijau (*L. sericata*) telah digunakan dalam terapi debridemen belatung untuk mempercepat penyembuhan luka sejak abad ke-19, setelah teramatinya galur bakteri yang resisten terhadap antibiotik. Ekstrak dari keseluruhan tubuh atau hemolimf larva lalat hijau diketahui memiliki aktivitas antibakteri. Penelitian ini bertujuan untuk menguji ekstrak etanol dari larva lalat hijau dalam mempercepat penyembuhan luka pada kulit tikus (*R. norvegicus*) betina. Metode penelitian adalah rancangan acak lengkap dengan enam perlakuan dan empat ulangan. Luka insisi sepanjang ±1,5 cm dibuat di bagian dorsolateral kulit yang telah dicukur menggunakan gunting steril. Ekstrak dengan konsentrasi 5, 10, atau 20% dalam minyak zaitun dioleskan pada bagian luka, sedangkan Betadine® digunakan sebagai obat pembanding. Pada hari ke-15, bagian luka diisolasi, difiksasi dalam larutan NBF 10%, ditanam di dalam parafin, disayat dengan ketebalan 5-7 µm, lalu dipulas dengan hematoksilin-eosin atau *trichrome* Heidenhain's Azan untuk pengamatan sediaan histologis. Hasil pengamatan menunjukkan bahwa perlakuan ekstrak larva lalat hijau konsentrasi 10% dapat mempercepat penyembuhan luka secara signifikan dengan mempercepat re-epitelisasi, mempersempit area jaringan granulasi, serta meningkatkan jumlah kapiler dan kepadatan kolagen dibandingkan dengan perlakuan lainnya ($p<0,05$). Berdasarkan hasil tersebut, ekstrak etanol larva lalat hijau dapat dikembangkan menjadi agensia untuk mempercepat penyembuhan luka pada kulit.

Kata kunci: ekstrak etanol, lalat hijau, ekstrak tubuh larva, luka insisi, penyembuhan luka

1. Introduction

The primary function of the skin is to serve as a protective barrier against the environment. Loss of the integrity of large portions of the skin as a result of injury or illness defined as wound.[1] In pathology, wounds remain a challenging clinical problem, with early and late complications presenting a frequent cause of morbidity and mortality.[2] Therefore, it is very important to restore skin integrity through the process of wound healing. The primary goals of the wound healing are rapid wound closure and a functional and aesthetically satisfactory scar.[1]

Wound healing is a dynamic set of tissue changes important for maintaining integrity of an organism, providing mechanisms by which injured tissue is prepared for reconstruction; it may be divided into four dynamic phases– vascular response, inflammatory response, proliferation, and maturation (remodeling)– that overlap in time.[1,3,4] Normal wound healing is a dynamic and complex process involving a series of coordinated events, including bleeding, coagulation, initiation of an acute inflammatory response to the initial injury, regeneration, migration and proliferation of connective tissue and parenchyma cells, as well as synthesis of extracellular matrix proteins, remodeling of new parenchyma and connective tissue and collagen deposition; involves multiple cell populations, the extracellular matrix and the action of soluble mediators such as growth factors and cytokines.[2]

A major factor that influences wound healing is bacterial infection. When a wound is infected by bacteria, it produces inflammation and accumulation of fluid. However, when an inflammatory stimulus cannot be eliminated or removed, this would leading to chronic inflammation which are more difficult to manage.[2] Non-healing wounds have a significant impact in public health and in the expenditure of public resources because the wounds can cause physical and psychological deficiency, or even death.[5] The increasing incidence of chronic wounds and their numerous socio-economic consequences have made wound management a key area of focus for researcher.[6]

Maggot debridement therapy (MDT), the treatment of suppurated skin infections with the larvae of calliphorids flies, was first introduced in USA by William S. Baer in 1931 was routinely used there until the mid-1940s in over 300 hospitals. With the introduction of antimicrobial, MDT became rare until the early 1990s, when it was re-introduced in the USA, UK, and Israel.[7] Larvae of the green-bottle fly (*Lucilia sericata*, Diptera: *Calliphoridae*) are increasingly used in MDT as a fast and effective treatment of necrotic chronic wounds. The application of sterile larvae to an infected non-healing wound results in the removal of necrotic tissue (debridement), disinfection, rapid elimination of infecting microorganisms, and enhancement of the healing process.[8] Larval therapy results in a reduction in wound pain, with relatively few side effects and cost-effective in comparison with conventional wound healing. The treatment also offer an efficient alternative to antibiotic therapy for the treatment of wounds contaminated with a variety of wound pathogens including methicillin-resistant *Staphylococcus aureus* (MRSA) and *Escherichia coli*.[6]

Various mechanisms by which larval debridement function in wounds have previously been suggested, including liquefaction of necrotic tissues by proteases, digestion of necrotic tissue as food by larvae, change in wound pH from acid to a beneficial alkaline pH by the excretion of ammonia and the irrigation of bacteria from the wound by serous exudate caused by the irritating effect of the larva in the wound.[7] The success of MDT has been credited to two of these modes of action: the disturbance of the wound and the secretion of proteases by the larvae such as trypsin-like and chymotrypsin-like enzymes and collagenase.[4,6]

The most commonly disadvantage of larval therapy is the negative perception which it is regarded by both patients and practitioners.[6] A study by Petherick *et al*. showed that approximately 25% of patients offered larval therapy for treatment of chronic leg ulcers would chose not to accept the

treatment, even if the maggots were confined to bags and therefore able to secrete enzymes and chemicals through the bag into the wound without being in direct contact with the patient.[9] Similarly, Spilsbury *et al*. found that patient compliance was not universal, with only 77% agreeing to leg ulcer treatment with maggots.[10]

Several study have shown that whole body extract of *L. sericata* contains antibacterial property, including against MRSA and *E. coli*.[11-13] In order to avoid negative perception, *i.e.* disgust feeling, in further application of the larva to human, we processed the larva to become an extract. In this study, we used the ethanol extract from whole body of the *L. sericata* larvae to examine the effectiveness against incision wound of rat skin. The rat skin structure is, in many aspects, similar to human skin.[14] The extract was applied immediately on the wounded site in order to avoid microbial infection that leading to chronic wound formation.

2. Methods

2.1. Instruments

The instruments were sterile dissection kit, rotary evaporator (Eyela N1100SWD), rotary microtome (Microm HM310), heating plate (Thermatic EDS-89), light microscope (Olympus CX-21), oven (Cole Palmer), and staining jar.

2.2. Materials

Twenty four male Wistar rats (160-180 g) provided by Biosystem Laboratory, Department of Biology were used. They were housed under standard environmental conditions of room temperature under a 12 h dark-light cycle, and allowed free access to drinking water and standard pellet diet (CP-551, PT. Charoen Pokphand). Rats were acclimated to holding facilities for one week prior to treatments.

The green-bottle larvae (*L. sericata*) were obtained from Balai Penelitian Tanaman Sayuran (Balitsa). All chemicals for extraction the larvae and histological preparation were purchased from Merck.

2.3. Experimental design

The experimental using completely randomized design with six treatments and four replications each. Animals were randomly assigned to control and treated groups, as described below.

1. Negative control 1 (NC1): non-treated wounded rats
2. Negative control 2 (NC2): wounded rats + olive oil as vehicle
3. LCE1: wounded rats + ethanol extract of *L. sericata* at concentration 5%
4. LCE2: wounded rats + ethanol extract of *L. sericata* at concentration 10%
5. LCE1: wounded rats + ethanol extract of *L. sericata* at concentration 20%
6. Reference (Ref): wounded rats + Betadine®

2.4. Whole body larval extract preparation

The larvae were air-dried to a constant weight and blend to a coarse powder. The dried powder was soaked and macerated on ethanol 95% (ratio 1:2) for 72 hours and every 24 hours the macerate was collected. The macerate was then evaporated using a rotary evaporator at a temperature of 40-50^{o}C to make a paste extract. The extract then diluted with pure olive oil to obtain concentration of 5, 10, and 20 % (w/v).

2.5. Procedure of the wound creation and treatment with the ointment

Before created of the wound, the rats were euthanized with an inhaled ether, the animals' back was shaved and the incision wound was made in the dorsal region with a ± 1,5 cm length using sterile scissors (full-thickness type extending up to the adipose tissue).[15] The treatment was immediately treated after the rats was injured, then given twice a day in the morning and afternoon for 14 days.

2.6. Histological preparation and examination

On the 15th days, after an overnight fast, the animals were weight and sacrificed by cervical dislocation, wounds site harvested and fixed in 10% neutral formalin buffer,

and processed in paraffin. For hematoxylin-eosin (HE) staining, we examined formation of re-epithelialization, granulation tissue, and number of blood capillaries. For Trichrome Heidenhain's Azan we examined collagen density. Histological examination was obtained in five areas per slide (each slide representing the respective rats).

2.7. Data analysis

Results were expressed as mean ± standard deviation (SD). Statistical significance for the number of blood capillaries was analyzed using one-way ANOVA followed by Duncan multiple range test, whereas the density of collagen was analyzed using Kruskal Wallis test followed by Mann-Whitney test. P values less than 0.05 were considered significant.

3. Results

During the post-surgery period, the animals remained healthy, without clinical evidence of infection. The microscopic observation also confirmed the aseptic conditions during the wounding in all groups. The process of wound healing can be observed morphologically with the skin closure at the wound site in the end of treatment period (Fig. 1). The histological examination of wound site in day 15th after treatment showed 100% re-epithelialization of all treated rats (Fig. 2). It was characterized by the formation of new epidermis layer above granulation tissue in wound site.

Histological observation on the wound site showed the area of granulation tissue, as well as blood capillaries and collagen density in as the parameter of proliferation process of wound healing (Fig. 2). The wounded rats of negative control group 1 (NC1) which is not treated and group 2 (NC2) which is treated by olive oil as vehicle had the most extensive granulation tissue, lower number of blood capillaries, and density of collagen which is indicated slower healing process than the other treatments. Application of ethanol extract of *L. sericata* larvae at concentration 5, 10, and 20% (LSE1, 2, 3) produced a narrower granulation tissue as well as an increased number of blood capillaries and density of collagen, along with the increased of the extract concentration. Application of Betadine® also produced narrower granulation tissue as well as an increased number of blood capillaries and density of collagen. We also observed the presence of hair follicles and sebaceous gland in the non-granulation tissue of wound site as a parameter of remodeling process in all treatment group.

Histological examination in wound site of LSE3 groups showed highest number of blood capillaries as well as score of collagen density that significantly different with other treatments ($p<0.05$). From all of the extract treatments, the wounded rats treated with the extract at concentration 20% (LSE3) showed better results compared with the reference

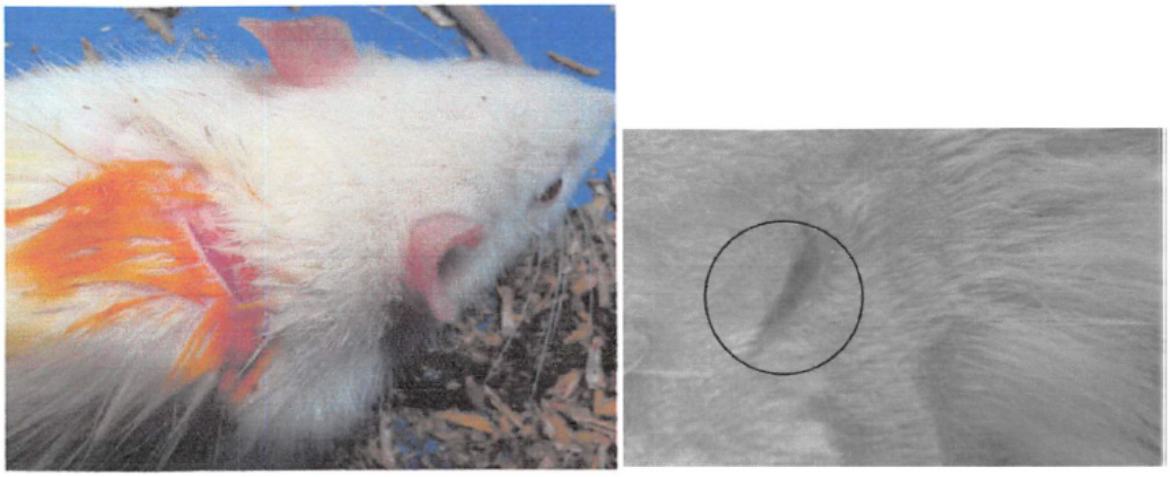

Figure 1. Photograph of wounded-rats before and after the treatment for 14 days.

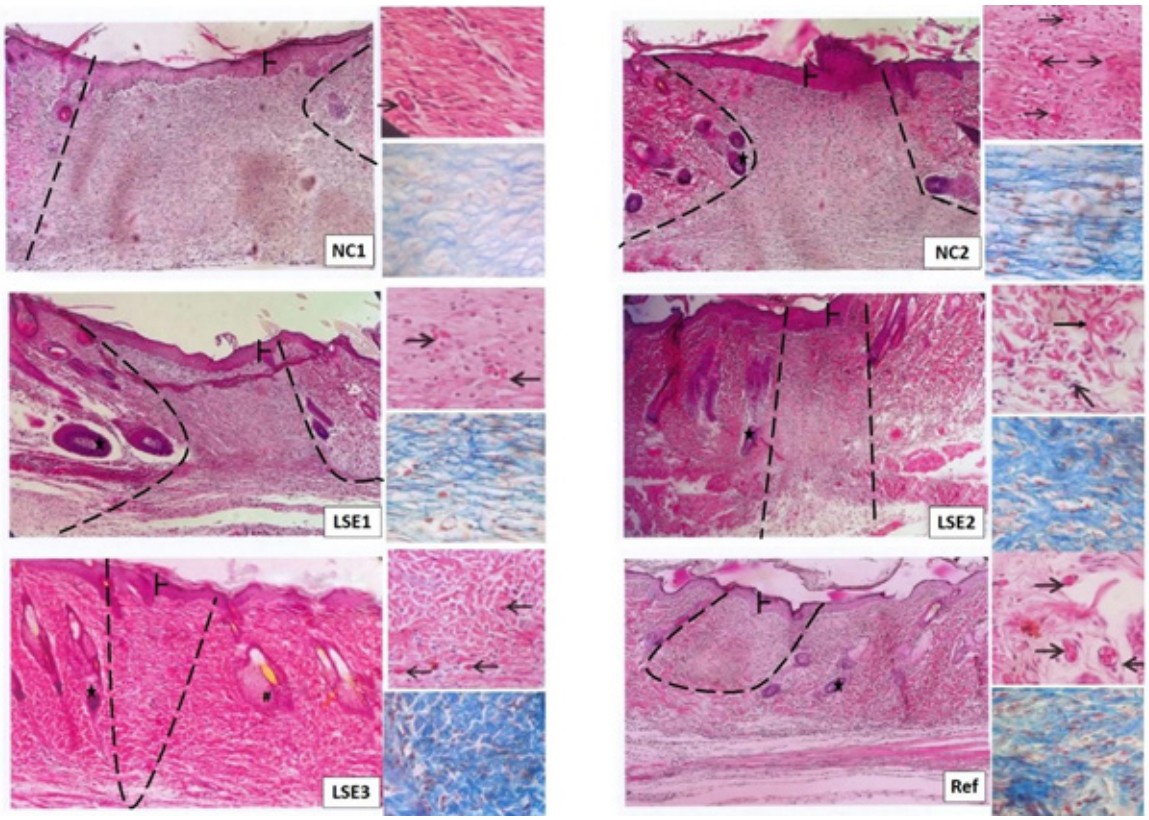

Figure 2. Photomicrograph of rat skin from each treatment groups after treatments for 14 days. Hematoxylin-Eosin or trichrome Heidenhain's Azan stain. M.100×.

Note: Epidermis in wound site indicated re-epithelialization (⊢) and granulation tissue area in dermis (bordered by dash line), as well as hair follicle (∗), and sebaceous gland (#) in non-granulation tissue. Inset (M.1000×): upper panel showed blood capillary (arrow) and lower panel showed collagen (blue) in granulation tissue of wound site.

group (Ref), thus could accelerate wound healing process in rat skin.

4. Discussion

Tissue injury causes the disruption of blood vessels and extravasation of blood constituents. Hemostasis of vascular response involves platelet coagulation and fibrin clot formation. The blood clot reestablishes hemostasis and provides a provisional extracellular matrix for cell migration. Platelets not only facilitate the formation of a hemostatic plug but also secrete several mediators of wound healing, such as platelet-derived growth factor (PDGF), that attract and activate macrophages and fibroblasts. After initial vasoconstriction at the wound site, there is an increase in vascular permeability that leading to inflammation signs, Inflammatory phase results in the active recruitment of leucocytes.[1,4]

Proliferative phase in wound healing process characterized by formation of epithelium (re-epithelialization), as well as blood capillaries (neovascularization) and collagen deposition in dermis layer. Re-epithelialization of wounds begins within hours after injury. Epidermal cells from skin appendages such as hair follicles quickly remove clotted blood and damaged stromal from the wound space. New stromal, often called granulation tissue, begins to invade the wound space approximately four days after injury. Numerous new capillaries endow the new stromal with its granular appearance.[1,2,4]

As the final phase of wound healing, the remodeling phase is responsible for the development of new epithelium and final scar tissue formation.[2] Collagen remodeling during the transition from granulation tissue to scar is dependent on continued synthesis and catabolism of collagen at a low rate, which is controlled by matrix metallo-proteinase secreted by macrophage, epidermal cells,

and endothelial cells, as well as fibroblasts. Wounds gain only about 20% of their final strength in the first three weeks, during which time fibrillar collagen has accumulated relatively rapidly.[1]

The beneficial effects of maggots on human wounds have been noted for years. This suggested that factor(s) from the maggot may be responsible for the rapid wound healing.[16] In this study, we used the ethanol extract from larval whole body. We assumed that the active compounds found in the whole body, haemolymph and secretions/excretions of the larva are similar and could accelerate the wound healing. Application of the extract could accelerate wound healing showed by narrower granulation tissue, increased number of blood capillaries and density of collagen compared with the control group and also better than the Betadine® application as a standard first-aid treatments for wound. Although, observation of re-epithelialization results cannot have confirmed which one of the treatment that accelerated on closure of the wound, because we only observed on 14th days. A study by Vidinsky *et al*. showed that re-epithelialization on skin wound in rats completely finished on five days after injuries.[14] Our *in vivo* study was support another study using extract of *L. sericata* whole body to accelerated wound healing.

In vitro study by exposing human fibroblast tissue culture to the *L. sericata* extracts showed that the extract could stimulated fibroblast growth. The studies suggest the existence of intrinsic factors within the maggot which may be responsible for the growth-stimulating effects in wound healing.[16] Furthermore, Parnes and Lagan observed that macrophages, fibroblasts, and blood vessels move into the wound space at the same time identification and isolation of molecules which underlie the proteolytic, antimicrobial and growth-promoting activities after application of the maggot.[4]

Recent scientific publications indicate the presence of distinct antibacterial and antifungal factors present in maggot secretions/excretions (SE). The antibacterial activity was observed in extracts of the whole body, haemolymph and in the excretions of maggots.[11] Maggot secretions fulfilled the required definitions of an antiseptic, and bactericidal activity of maggots was effective against *Micrococcus luteus*, *S. aureus* both a methicillin-sensitive strain and a methicillin-resistant strain (MRSA), and *E. coli*.[12] Secretions of *L. sericata* also shown antifungal activity against *Fusarium sambucinum* and *F. verticillioides*.[17]

Larval secretions of *L. sericata* also contain a variety of alkaline compounds including ammonium carbonate, calcium, allantoin and urea that inhibit bacterial growth. The presence of secretions resulted in a reduction in fibroblast adhesion to

Table 1. Number of blood vessels and collagen density after 14 days of the treatment in wounded-skin of rats

ID	Treatments	Number of blood capillaries*	Score number of collagen density**
NC1	non-treated wounded rats	3.75 ± 1.25 **(b)**	2.50 ± 0.58**(a)**
NC2	wounded rats + olive oil as vehicle	4.48 ± 2.51 **(ab)**	2.50 ± 0.58 **(a)**
LSE1	wounded rats + ethanol extract of *L. sericata* at concentration 5%	5.32 ± 2.58 **(ab)**	2.50 ± 0.58 **(a)**
LSE2	wounded rats + ethanol extract of *L. sericata* at concentration 5%	7.54 ± 0.57 **(b)**	3.25 ± 0.50 **(ab)**
LSE3	wounded rats + ethanol extract of *L. sericata* at concentration 5%	13.25 ± 2.36 **(c)**	3,75 ± 0.50 **(c)**
REF	wounded rats + Betadine®	6.87 ± 0.81 **(b)**	3,00 ± 0.00 **(a)**

Note: The value is expressed as mean ± standard deviation (n=3). *Data were analyzed using one-way ANOVA followed by Duncan multiple range test. ** Data were analyzed using Kruskal Wallis followed by Mann-Whitney test. Difference alphabet in the same column showed $p<0.05$ and considered significant.

fibronectin and collagen. Larval secretions has proteolytic activity which can altering the structure of the extracellular matrix. The behavioral modification within the wound may promote the formation of new tissue, because the activity of trypsin-like and chymotrypsin-like proteinases strongly influenced the remodeling of the extracellular matrix.[6] Telford *et al*. have produced an active recombinant chymotrypsin I isolated from the larvae of *L. sericata* which has greater enzymatic activity when compared to mammalian chymotrypsin, and the chymotrypsin degrades ECM components in wounds and aids fibroblast migration through this action.[18]

The antimicrobial factor in *L. sericata* that is effective against pathogenic elements of the wound microbial flora was lucifensin, which purified from the extracts of various tissues (gut, salivary glands, fat body, and haemolymph) *L. sericata* larvae and from their excretions/secretions. Another compounds with antimicrobial activity other than lucifensin also detected in the fat body.[8] Lucifensin expression was strongly stimulated in the fat body by the presence of *S. aureus* and *Pseudomonas aeruginosa.*[13] Zhang *et al*. extracted unsaturated fatty acid from dried *L. sericata* larvae which can promote murine cutaneous wound healing, by up-regulating transcripts and protein of VEGFA expression and increasing the amount of new-formed capillary at inflammatory phase as well as the percent wound contraction at granulation formation phase and scar remolding phase.[19] Towards next generation of maggot application was the transgenic *L. sericata* larvae that produce and secrete a human growth factor because of transgene insertion that express human PDGF-BB.[20]

It is concluded that the treatment of *L. sericata* extract immediately on skin wound may have a beneficial influence by inhibit the microbial infections, thus accelerated the wound healing process by enhanced re-epithelialization and granulation tissue, as well as increased capillary number and collagen density of wound site in rat skin.

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