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Lucilia sericata Larval Secretions Stimulating Wound Healing Effects on Rat Dermal Fibroblast Cells

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Abstract: The extract from larval *Lucilia sericata* is used clinically to promote wound healing and tissue regeneration. However, its effect and underlying mechanisms on fibroblast cells, which are involved in the wound healing process, are still poorly understood. This study aimed to examine the effects of larval secretions on dermal fibroblast activity and gene expression and to evaluate the wound healing potential of their major components. Primary rat fibroblasts were cultured and treated with larval secretions. Following the treatment, the cells were used to extract RNA for gene profiling. In addition, migration to the injury site was studied with the scratch healing assay. Our results showed that larval secretion accelerated the migration of the fibroblasts compared to the control cells and that several mRNAs were differentially expressed during a period of 72 h incubation. Additionally, we analyzed the chemical composition of larval secretions and showed that fumaric acid, ferulic acid, and *p*-coumaric acid, which were selected and identified for their major components, enhanced the migration of the fibroblasts. Therefore, these results indicate that *L. sericata* larval secretions could modulate the mRNA expression of some wound healing-related genes of the fibroblasts and contain the effective components for wound healing.

Keywords: *L. sericata* larval secretion; wound healing; fumaric acid; ferulic acid; LC-HRMS, GC-MS.

1. Introduction

Lucilia sericata larvae have been used as an alternative approach in the treatment of many chronic and necrotic wound types, including diabetic ulcers, post-surgical wounds, and burns. Maggots are used for debridement of necrotic tissue for hard to heal wounds, disinfection of the wound, and to stimulate the regeneration process by proteolytic digestion [1]. The larvae have mechanical and biochemical roles such

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as digestion of necrotic tissue, suppressing inflammation, and promoting granulation tissue in wounds [2]. However, the molecular mechanisms underlying larval treatment mediated wound healing are not yet sufficiently explained and still need to be investigated in detail. The beneficial effect of larval secretion (LS) in wound healing probably occurs by the synergistic action of different bioactive molecules, including a large variety of growth factors and digestive enzymes, which are secreted by maggots. Application of LS on a wound of rat skin modulates several genes which are important proteins contributing to the wound healing process [3]. They observed that maggot treated groups have shown rapid and better collagenation, epithelialization and neovascularization with perfect healing of the wound in two weeks. The maggot effects on healing when used alone or in combination with antibiotics were recorded as similar. It was demonstrated that the maggots of *L. sericata* possess a definite antibacterial action along with the removal of dead tissue and effectively reduced the bacterial bioburden in the infected wound and induced wound healing quickly [4]. It was also suggested that *L. sericata* in the wound ingest necrotic tissue, sparing healthy tissue and also ingest bacteria and thereby increase oxygen perfusion, rapid spread of granulation tissue, cellular proliferation, fibroblast migration and matrix remodeling [5]. Another reason for faster wound healing in the maggot treated groups might be because the application of maggots promotes stimulation of angiogenesis which is one of the complex series of events in wound healing and thereby causes the proliferation of endothelial cells in the injured area [6].

Traumatic effects on dermal tissue cause different degrees of a tissue defect, necrosis, and degeneration. The cutaneous wound healing is a well-regulated process with a complex regenerative response after tissue damage involving growth factors, extracellular matrix, and components of cells. Therefore cell proliferation and ECM (extra cellular matrix) remodeling should appropriately be conducted for tissue repair. Fibroblasts are one of the most important cell types in the wound healing process. There are several genes expressed by fibroblasts during different phases of wound healing, all of which show critical potential in effective healing. Fibroblasts secrete proteins that are used for the reorganization of ECM. Proliferation and migration of fibroblasts from the edges of the wound sites are necessary for granulation and regeneration of the wound. The migration of fibroblast cells to the wound site facilitates the synthesis of growth factors, in which extracellular matrix synthesis takes place by the migration of other cell types to the wound site [7].

It has been demonstrated that maggot secretion stimulates the proliferation and migration of fibroblasts. Also, with the addition of epidermal growth factor, maggot secretions significantly increased fibroplasia [8]. Other evidence demonstrated that maggot secretions significantly promoted the migration of epidermal keratinocytes and fibroblasts during wound closure [9]. It was demonstrated that LS include proteinases, especially serine proteinases, which promote fibroblast migration. It is suggested that maggot secretion promoted cell migration of human dermal fibroblasts, which is related to the degradation of fibronectin on the cell surface [10]. To understand the mechanism behind the effect, an in-vivo model was developed to examine cellular morphology and fibroblast migration in response to maggot secretion. This model, which simulates the microenvironment *in-vivo*, provided information on the interactions between the LS, resident cells, and ECM in the wound healing process. Further incubation with LS, metabolic activity, and protein expression was increased to build the microfibrillar structure which was essential for cellular migration and ECM remodeling [11].

Based on the discussed literature and LS-induced gene expression changes in the wound environment obtained from our previous study [12], it may be hypothesized that LS may have a significant effect on the gene expression of fibroblasts as related to the wound healing process. Hence in this study, we analyzed the expression of 84 genes associated with wound healing in LS-treated primary rat fibroblasts in the context to the wound healing potential. Second, we also evaluated the in vitro wound healing potential of components of larval secretion, ferulic acid, fumaric acid, and *p*-coumaric acid, found in significant amount in LS, following chemical characterization of their active components by GC-MS and LC-HRMS. This study may help better understand the wound healing effect of LS on dermal fibroblast activity and gene expression.

2. Materials and Methods

2.1. Preparation of the Lucilia sericata Larval Secretion

Larval secretion was extracted in sterile pure water using the second and third instar larvae of *Lucilia sericata*. LS was pooled after incubating approximately 2000 larvae at room temperature. After, 5 mL sterile water was added at 1 h intervals for 5 h, while the LS secreted into the water. LS was collected and centrifuged at 1500 g for 10 min to remove unwanted particles. The protein concentration of LS was measured using the Bradford method.

2.2. Cell Isolation and Culture

Primary rat dermal fibroblasts were isolated from skin biopsies from 4 week old Wistar rats. Rats were sacrificed after they were anesthetized with 5% isofluorane. After shaving the skin 1 cm^2 patches were isolated. Then, patches were sliced into small pieces and were transferred to 90 mm Petri dishes. After this D-MEM cell culture medium was supplemented with 10% FBS, penicillin (10 units/mL) and streptomycin sulfate (10 μg/mL) (GIBCO) at 37°C and 5% CO_2 in an incubator. The medium was changed every 3 days. After reaching 90% confluency, cells were trypsinized, transferred into T25 culture flasks, and cultivated again to expand to around 70% confluence [13]. Cells were used within passage 4–6. Animal studies were done following the instructions of the Local Ethics Committee of Bezmialem Vakif University Experimental Animal Studies (Reg. No. 874.2016-243, See supporting information S1).

2.3. Scratch Assay

The rat fibroblast cells were cultured in 12 well plates. Around 3×10^4 cells were seeded to each well and were allowed to reach 90% confluency. Using a 200 μL tip, the cell monolayers were scratched and rinsed with PBS to remove detached cells and other debris [13].

2.3.1. Analysis of L. sericata Secretion

The culture medium was replaced with fresh media supplemented with *L. sericata* secretion 50 μL (protein concentration of 50 μg/mL) and the plates were incubated at 37°C with 5% CO_2. Three representative images from each of the scratched areas were photographed to estimate the relative migration cells. The distance between the two edges of the wound sites was detected at 0, 12, 24, and 48 h and analyzed by Image J software. Wound closure was calculated using the formula: Wound closure (%) = [(wound site day O - wound site in the indicated day)/wound site day O] × 100 [14].

2.3.2. Analysis of Chemical Ingredients

The scratched cell culture medium was replaced with fresh media supplemented with 100 μM fumaric acid (95%, Sigma-Aldrich), 100 μM *trans*-ferulic acid (97%, Sigma-Aldrich) and 100 μM *p*-coumaric acid (98% Sigma-Aldrich) separately, and the plates were incubated at 37°C with 5% CO_2. The control was treated with 0.1% DMSO only. The distance between the two edges of the wound sites was detected at 0, 24, and 48 h and analyzed by the wound closure formula.

2.4. RNA Extraction and cDNA Synthesis

Total RNA was extracted using a trypsinized GeneJET RNA Purification Kit (Thermo Fisher Scientific, USA) from cultured cell lysate. The integrity of the extracted RNA was analyzed by 1.5% agarose gel electrophoresis. Quantity and purity of the RNA were analyzed by spectrophotometry, absorbance at

260 nm and 280 nm using the Thermo Scientific Multiskan GO (Thermo Fisher Scientific, USA). 1 µg of total RNA was used for cDNA synthesis using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) following the manufacturer's instructions.

2.5. PCR Array and Quantitative PCR

PCR reactions were performed using Rat Wound Healing RT2 Profiler PCR Array (Qiagen, Germany) containing specific primers for 84 genes related to wound healing. For the normalization of gene expressions, five different housekeeping genes, actin beta, beta-2 microglobulin, hypoxanthine phosphoribosyl transferase 1, lactate dehydrogenase A, ribosomal protein large P, and blank wells for negative control were included. Included genes in the PCR Array are listed in Table S1. For the reaction enzyme and mixture, iTaq Universal SYBR Green Supermix (Bio-Rad) was used. After the addition of the cDNA template, according to the manufacturer's instructions, the reaction mixture was loaded into the 96-well PCR array plate. Next, the reaction was performed using a CFX Connect Real-Time PCR Detection System (Bio-Rad). PCR conditions were as follows: initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation for 15 s at 95°C, annealing for 20 s at 60°C and extension for 30 s at 72°C.

2.6. Evaluation of Quantitative PCR Data

The relative expression and fold change of mRNA were calculated with the $2^{-\Delta\Delta CT}$ method. The expression results were presented as a significant upregulation or downregulation in the mRNA level. RT^2 Profiler PCR Array Data Analysis Web Portal was used for the data analysis (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php). The differential expression levels between LS treated fibroblast cells and controls were analyzed using the $2^{-\Delta\Delta Ct}$ method. ΔCt indicates the difference in expression levels with the Ct value of the target gene and means of five housekeeping genes (ΔCt = Ct target gene – Ct housekeeping), and ΔΔCt indicates the difference in the ΔCt value between treatment and control groups (ΔΔCt = ΔCt LS – ΔCt control).

2.7. Gas Chromatography Mass Spectrometry Measurements

10 mL of LS was lyophilized in a Christ lyophilizator. 100 mg of the lyophilized LS was weighed and mixed with 100 µL of dry pyridine and 100 µL bis (trimethylsilyl) trifluoroacetamide (BSTFA) and heated at 105°C for 45 min, and then, the final supernatant was analyzed by gas chromatography–mass spectroscopy (GC–MS) [14]. The composition of LS was analyzed using a ThermoScientific Gas Chromatography mass spectrometer. A DB-5 capillary column (30 m, 0.25 mm, 0.25 µm film thickness) was used. The carrier gas was helium at a rate of 1 mL/min. GC oven temperature was kept at 80^0C for 5 min and programmed to 300^0C at a rate of 4^0C/min and kept constant at 300^0C for 5 min. The injection temperature and source temperature were 250^0C and 220^0C, respectively. MS interface temperature was 240^0C. The injection volume was 0.5 mL with a split ratio of 1:30. EI/MS were taken at 70 eV ionization energy. Mass range was from *m/z* 50 to 650 amu, scan time was 0.5 s with 0.1 interscan delays. The library search was carried out using NIST and Wiley GC/MS library and İLMER library. ULTRAWRK 101 and 102 hydrocarbon mixtures were used for the comparison of the GC chromatograms. The relative percentage of separated compounds was calculated from the Total Ion Chromatography by the computerized integrator.

2.8. Liquid Chromatography High Resolution Mass Spectrometry (LC-HRMS) Measurements

LC-HRMS measurements were performed with a Thermo Orbitrap Q-Exactive instrument in ESI Source and equipped with a Fortis C18 column (150 mm x 3mm, 3 µm particle size) with a slightly modified method of literature [15]. The mobile phase was composed of water (A, 0.1% formic acid) and methanol (B, 0.1% formic acid), the gradient program of which was 0-1.00 min 50% A and 50% B, 1.01-05.00 min 100% B and finally 60-10 min 50% A and 50% B. The flow rate of the mobile phase was 0.30 mL/min, and

the column temperature was set to 24°C. The injection volume was 10 µL. As described in the literature the best mobile phase solution was determined to be a gradient of acidified methanol and water system. The good ionization of small and relatively polar secondary metabolites was obtained by the ESI source. The ions between *m/z* 85-1500 were scanned in high resolution mode. Identification of compounds was done by comparison of retention time of standard compounds and HRMS data of the Bezmialem Vakif University, Drug Application and Research Center Library (İLMER). Freshly prepared dihydrocapsaicin (purity 95%) was used as an internal standard (See Figure S1).

2.9. Statistical Analysis

Data are expressed as the mean ± S.E.M. and statistical analyses were performed using one-way ANOVA SPSS version 21.0 software (SPSS, USA). Significant differences between the treated groups and the control were determined by Student's t-test, at a level of $P < 0.05$. Method validation and uncertainty evaluation applied based on literature and the summary is given in section 3 [16-20].

3. Results and Discussion

The current study investigated the role of *L. sericata* LS in modulating gene expression at the transcriptional level on a fibroblast cell, which is one of the major cell populations involved in the wound healing process, and evaluated the in vitro wound healing potential of three molecules found in significant amounts in LS.

3.1. The Effects of LS Treatment on Gene Expression

The healing potential of rat skin fibroblast cells following 12, 24, and 48 h exposure to LS was analyzed by the scratch assay, which is one of the most used methods and which supports the understanding of wound healing efficacy of therapeutic agents.

After scratching the confluent fibroblast monolayer, it was observed that the cells with LS promoted the migration into the scratched site and closed the empty area (Figure 1 A). The LS promoted wound closure compared to the negative control. At 12 h, the cell migration rate for the treatment group was higher (28.2 ± 3.1) compared to the control group (14.5 ± 3.8). At 24 and 48 h, the migration rate towards the wound area was again higher in the treatment groups (62.0 ± 2.8%, 48.1 ± 5.6% and 87.3 ± 4.1%, 81.9 ± 3.7% respectively) than the control group (Figure 1 B). Therefore, this result shows that LS has an enhancing effect on the migration and the spreading of fibroblasts, which are one of the major determinants of the wound closure rate.

On the other hand, to investigate whether there is a role in modulating gene expression of LS in the fibroblast cells, primary rat fibroblasts were cultured with LS for 72 hours and compared with the differential expression of 84 genes mRNAs related to wound healing between LS treated fibroblasts and controls by using quantitative RT-PCR array with primers specific for genes encoding extracellular matrix components, cellular adhesion molecules, signal transduction proteins, cytoskeleton proteins as well as inflammatory cytokines and chemokines (Table S1).

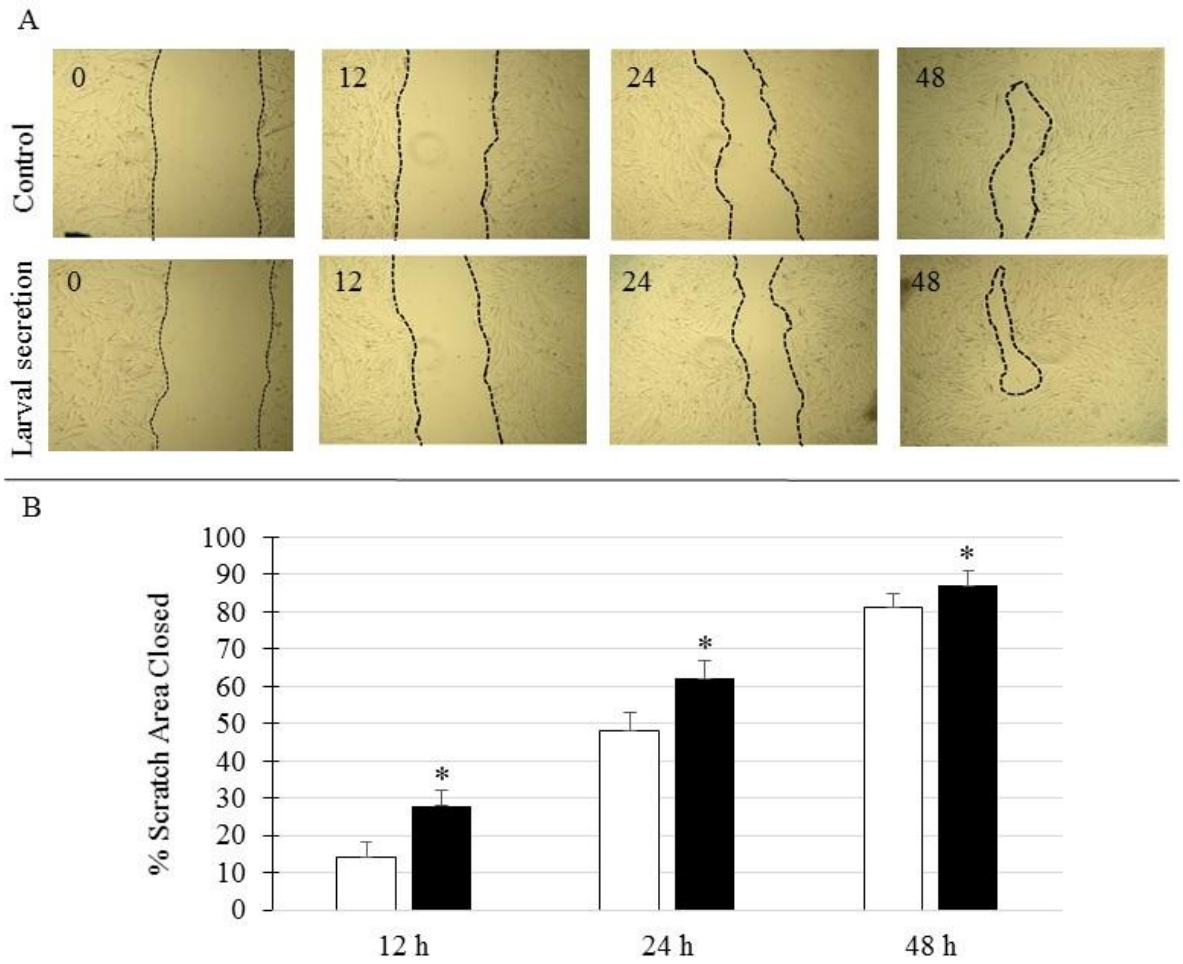

Figure 1. Wound closure effect of LS. Light microscope images of wound closure using fibroblast cells. **A**: The images show the cell migration into the scratched wound area in the presence of LS and the control. Images were captured at 0, 12, 24, and 48 h and compared with controls. **B**: Scratch closure was analyzed with the measurement of the empty scratched area. Each time point was compared to the image on day 0 and reported as percent closure. Data are presented as the mean±SD of 3 repeats per group. *p < 0.05 compared with the untreated control. White bars: Control, black bars: Larval secretion.

The gene profiling results revealed that 9 out of 84 mRNAs were differentially expressed in the presence of LS in cultured fibroblasts. Differential expression over 4-fold was evaluated as significant after comparison to the controls (Table 1). The list in Table 1 shows the gene abbreviation and description, the up or downregulation, and the p-value. LS-treated dermal fibroblasts significantly up-regulated expression of Col14a1 (Collagen, type XIV, alpha 1), Cxcl11 (Chemokine C-X-C motif ligand 11), Ctsl1 (Cathepsin L1), Fgf7 (Fibroblast growth factor 7), Hgf (Hepatocyte growth factor) and Il10 (Interleukin 10) in a time-dependent manner. Col4a1 was the highest upregulated (13.89-fold) among the 5 upregulated mRNAs. In contrast, mRNA levels for Itgb6 (Integrin, beta 6), Tnf (Tumor necrosis factor superfamily, member 2), Fgf2 (Fibroblast growth factor 2) were downregulated in the LS treated fibroblasts. Tnf had the lowest fold change (8.31 fold) among three downregulated mRNAs (Figure 2).

Table 1. Differentially expressed genes after 72 h incubation in LS treated fibroblasts compared to untreated controls.

Up and downregulated genes	Fold change	*p*-value
Col4a1	13,89	0.0342*
Cxcl11	4,89	0.0453*
Hgf	7,86	0.0048*
Fgf7	11,93	0.0282*
Il10	12,96	0.0115*
Ctsl1	6,84	0.0271*
Itgb6	-6,54	0.0317*
Tnf	-8,31	0.0418*
Fgf2	-5,29	0.0394*

Fold change of the gene expression calculated with the $2^{-\Delta\Delta Ct}$ method. Asterisks (*) indicate statistical significance ($p < 0.05$). Col14a1(Collagen type XIV alpha 1), Cxcl11 (Chemokine C-X-C motif ligand 11), Hgf (Hepatocyte growth factor), Fgf7 (Fibroblast growth factor 7), Il10 (Interleukin 10), Ctsl1 (Cathepsin L1), Itgb6 (Integrin beta 6), Tnf (Tumor necrosis factor member 2), Fgf2 (Fibroblast growth factor 2).

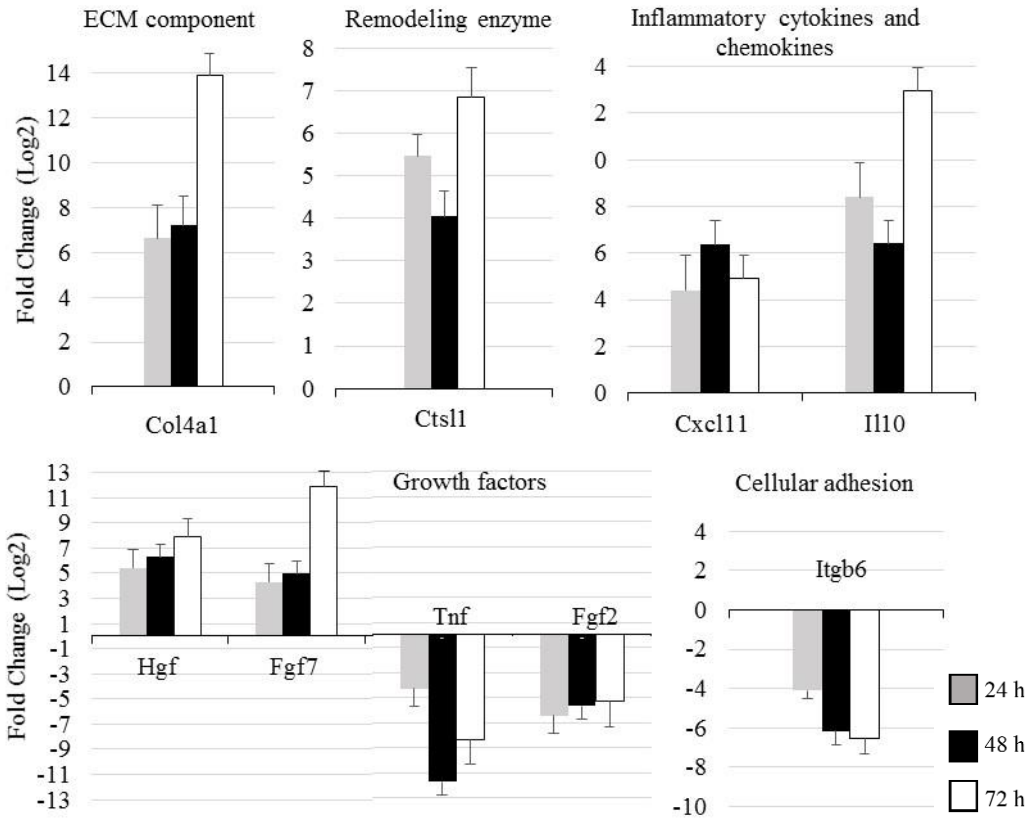

Figure 2. The differential mRNA expression of wound healing markers in fibroblast cells. Fibroblasts are treated with LS and compared with controls after 24, 48, and 72 h of incubation. All groups showed significant differences by one-way ANOVA test ($p< 0.05$). Expressions of the genes were normalized to the five different housekeeping genes.

Collagen synthesis and regulation is a very important event occurring at the maturation stage of wound healing. The *Col14a1* gene, which is among differentially expressed genes for LS treatment, makes the alpha1(IV) chain of type IV collagen [21]. It is suggested that the upregulation of Col4a1 is critical and is necessary during normal wound closure. In the absence of such up-regulation, wound healing is severely impaired [22]. In a rat model of the acute skin defect, it was shown that LS significantly changes Col4a1 expression [12]. The literature reported that Col4a1 expression was upregulated over 1000-fold on the 4th day during the early stage of wound healing. At the following stages, expression was gradually decreased but still high around 150-fold on day 10. In our in vitro conditions, Col4a1 expression with LS treatment gradually increased up to 14-fold during a period of 3 days. Thus, the up-regulated Col4a1 expression in dermal human fibroblasts exposed to LS is correlated with the in vivo data.

Chemokines secreted by different cell types including fibroblasts are small regulatory molecules that control the migration of cells to the wound tissue. It is known that cultured human fibroblasts from various tissues and pathologic settings produce a distinct panel of chemokines. In wound healing, chemokines are essential factors involved in controlling angiogenesis at different stages. CXCL11, which is a principal ligand in the CXCR3 signaling pathway for wound healing, stimulates re-epithelialization, and regulates the maturation of the superficial dermis [23]. It has been proposed that fibroblast secreted CXCL11 in response to injury may have a potential effect on quicker wound healing without scarring [12]. The present study shows that LS is effective in increasing Cxcl11 gene expression up to 4.89-fold in the fibroblast cell culture. Therefore, our data can provide indirect evidence suggesting that CXCL11 induction by LS treatment can improve wound healing. However, this question requires further study.

Growth factors known to be synthesized by fibroblasts play important roles in different stages of the wound healing process. Among them, hepatocyte growth factor (HGF) is a potent growth factor with mitogenic, angiogenic, motogenic, anti-fibrotic, and anti-inflammatory functions in different cell types. HGF is a growth factor with a pleiotropic effect, potentially accelerates wound healing and prevents the formation of fibrosis [24]. HGF can accelerate wound healing by controlling the dedifferentiation of skin cells in a process related to the β1-integrin and integrin-linked kinase signaling pathways [24]. The results of our study are consistent with the literature with HGF expression increasing gradually in three days up to 7.8-fold. Fibroblast growth factor 7 (FGF7) is a well-known factor reported to promote the proliferation of mesenchymal stem cells of skin tissue during wound healing [25]. The literature reported that FGF7 expression up-regulated 160 times after cutaneous damage in rat excisional wounds. It has been also shown to specifically stimulate the proliferation and migration of keratinocytes [26]. Thus, it has a pronounced effect on re-epithelialization of fibroblast cells, which have a crucial role during the second phase of wound healing between 2 and 10 days after injury. This phase is characterized by the migration and proliferation of different cells [27]. The FGF7 is expressed exclusively by dermal fibroblast cells at the wound site, and help wound re-epithelialization. It was also found in our study that FGF7 expression was significantly overexpressed up to 12-fold after 72 h in the LS treated fibroblasts.

TNF-a, which is among down-regulated genes by LS treatment, is functional during the early inflammation period and not expressed during the re-epithelialization and ECM remodeling stages of wound healing. TNF-a can promote wound repair by indirectly increasing growth factors derived from a macrophage cell and inducing inflammation. However, at higher levels, TNF-a causes a harmful effect on healing, through catabolic activity, which increases the expression of MMPs that degrades the extracellular matrix. subsequently preventing cellular migration and collagen accumulation [28]. In pathological courses, like in chronic wounds, the wound healing cascade is damaged. Wounds remain in the inflammatory state and are exposed to abundant neutrophil infiltration which produces inflammatory cytokines (TNF-a) and a high level of reactive oxygen species [29]. IL-10 is a major regulator in suppressing the inflammatory response, which plays a crucial role in the wound healing process. IL-10 is a well-known anti-inflammatory factor that restrains the activities of proinflammatory cytokines, like IL-1, IL-6, IL-8, and TNFα. It has been shown that LS could decrease the production of proinflammatory TNFα, but inversely increases the expression of IL-10. The increments of TNFα levels lengthen the inflammatory phase during the wound healing process [30]. The increased levels of IL-10 potentiate the activity of macrophages in the wound area and stimulate the wound healing process [31]. Our results are very consistent with this in vivo experiment.

In our results, while there was a significantly increased expression for the IL-10 (12.96-fold), the expression of TNFα was downregulated with LS-treatment (8.31-fold) in the fibroblasts. Therefore, our in vitro study on the dermal fibroblast could also contribute to explain in vivo observed findings.

LS also increased the expression of Ctsl1 gene coding for cathepsin L protein. Cysteine cathepsins are proteases localized in lysosomes and catalyze the degradation of a large number of proteins and have important functions in different physiological processes such as inflammatory activities, protein clearance, and signaling cascades [32]. By degrading collagen molecules, they help wound healing by promoting the migration of keratinocytes and fibroblasts and by stimulating endothelial cell angiogenesis [33]. Ctsl1 also plays a critical role in TNF-a induced cell death. The expression of Ctsl1 is significantly elevated in different types of inflammatory and neoplastic tissues [34]. However, limited data exist assessing the effect of ctsL1 in wound healing. In our study, treatment with LS increased (6.8-fold) the expression of ctsl1 in dermal fibroblast cells. More studies are necessary to clarify why the Ctsl1 transcript is more expressed in LS-treated fibroblasts.

In the present study, LS treatment of our cultured dermal fibroblasts also produced a significant decrease in the expression of the integrin αvβ6. Integrins are surface proteins that mediate signal transmission between the extracellular and intracellular environments. During transmembrane signal transduction, integrin αvβ6 interacts with both intracellular and extracellular molecules [35] and αvβ6 is produced only by epithelial cells, but is absent in normal healthy adult tissue or is expressed in small amounts [36]. In contrast, it increases in various types of inflammation, wound healing, fibrosis, during development, and multiple cancers. Each of these processes is required for tissue remodeling [37].

Also, there is experimental evidence showing that the level of αvβ6 integrin is increased significantly in wound healing. From current literature, it is difficult to explain why down-regulation of αvβ6 mRNA occurred in LS-treated dermal fibroblasts in context to enhanced cell migration observed in our study. The proposed mechanism of action of the differentially regulated genes in the presence of LS on fibroblast cells is given in Figure S3.

3.2. Chemical Composition and Wound Healing Activity

In the second part of the study, we aimed to determine the active and responsible ingredients of LS for wound healing by investigating their impact on early healing mechanisms associated with cell migration in primary cultures of rat dermal fibroblasts.

By GC-MS analyses, we have first defined that the main components of secretion are composed of waxy hydrocarbons. The major components were hexacosane (25.4 %), pentacosane (20.2 %), hentriacontane (15.8) and tetracosane (13.4 %) (Table 2 and Figure S1).

In addition to waxy components, the determination of secondary metabolites in LS was done by using LC-HRMS. Fumaric acid was identified as the main component of LS with a 3.8±0.1 mg/L concentration. *Trans*-ferulic acid (1.4 ±0.3 mg/mL) and *p*-coumaric acid (0.8±0.1 mg/L) were also quantified in the larval secretion. In addition to these compounds, caffeic acid was also determined as a trace amount in LS. Validation data of LC-HRMS- e.g. target ions, linear regression equations, the correlation coefficients (R^2), LOD/LOQ and Recovery %- are given in Table 3. The chromatogram of quantified ions is given in Figure S2 as supporting information. Detailed procedures of the uncertainty of evaluation are also available in the literature [16-20].

Table 2. Identified compounds in larval secretion by GC-MS

Compound	RI	%	Molecular Formula
Hexadecane	1600	0.6	$C_{16}H_{34}$
Phytan	1811	2.6	$C_{21}H_{44}$
Heneicosane	2100	1.1	$C_{21}H_{44}$
Docosane	2200	2.1	$C_{22}H_{46}$
Triacosane	2300	5.7	$C_{23}H_{48}$
Tetracosane	2400	13.4	$C_{24}H_{50}$
Pentacosane	2500	20.2	$C_{25}H_{52}$
Hexacosane	2600	25.4	$C_{26}H_{54}$
Hentriacontane	3100	15.8	$C_{31}H_{64}$
Hexatriacontane	3600	7.1	$C_{36}H_{74}$
TOTAL identified	94.0	94.0	

*Standard deviation of 3 replicates is ± 0.01

Table 3. Compounds determined in larval secretion by LC-HRMS and validation data

Compound	*m/z**	Linear Regression Equation	R^2	LOD/LOQ	Recovery %	mg/L
Fumaric acid	115.0037	$y=1.855.10^{-3}X + 5.312.10^{-4}$	0.997	0.26/0.88	97.27	3.8±0.1
t-Ferulic acid	193.0496	y =3.483x-0.064	0.981	0.11/0.33	95.6	1.4 ±0.3
p-Coumaric acid	163.0473	y = 8.126x-0.008	0.992	0.20/0.60	95.6	0.8±0.1
Caffeic acid	179.0350	$y=1.68.10^{-2}X + 5.922e-3$	0.999	0.19/0.62	102.3	<LOQ

*Negative ions

It has been known that cuticular hydrocarbons are one of the components of the lipid wax layer of *Lucilia sericata* as well as all insects [38, 39]. It is described that its main function is to prevent both desiccation and the penetration of pathogens. Our study showed that this component also exists in LS of *Lucilia sericata*. These waxy hydrocarbons are also present in some plant tissues. It was reported that leaf extract of *Moringa oleifera* and *Ficus asperifolia* includes a high amount of hexacosane, pentacosane, and heptacosane which are presents antioxidant capacity and antimicrobial activity [40, 41]. It may be speculated that the waxy hydrocarbon content of LS contributes to wound healing by an antibacterial and antioxidant barrier. However, it is unclear from the literature whether hydrocarbons in the LS are effective in fibroblast migration or proliferation and what are their potential roles on the wound healing process. Further in-vivo and in-vitro experiments need to be done to demonstrate the probable effect of waxy hydrocarbons in wound healing.

Reports on the experiments with different designs indicate that the associated major acidic compounds found in LS have wound healing activity, which may be due to various properties such as anti-oxidant, anti-bacterial, and anti-inflammatory. However, to our knowledge, their role on the fibroblast cell in relation to wound healing has not been defined. Hence, we tested the wound healing capability of three major components from among the quantified compounds of LS by LC-HRMS to better understand the role of its components on the fibroblasts in the context of wound healing.

Taking into account the information in the literature, the concentration selected for the study was 100 µM for all compounds, because it was in the optimum range for the observed effects in in-vitro studies with different designs and was confirmed to have no cytotoxic effects on cells. When we performed scratch assay on the rat primary fibroblast cells treated with each test compound for 48 h, our results indicated that all of

the tested compounds significantly ($p<0.001$) stimulates wound healing compared to control samples in 48 h. In this assay, *t*-ferulic acid and fumaric acid, treatment also stimulates wound healing in 24 h, while *p*-coumaric acid treatment does not improve wound healing during this time period. The extent and rate of wound closure for fumaric acid was much faster compared to other tested compounds. It closed the gap created by scratch by approximately 89% compared with the control treatment in 48h. This result showed that fumaric acid was the most effective compound among ones tested in increasing the migration rate of fibroblast cells (Figure 3).

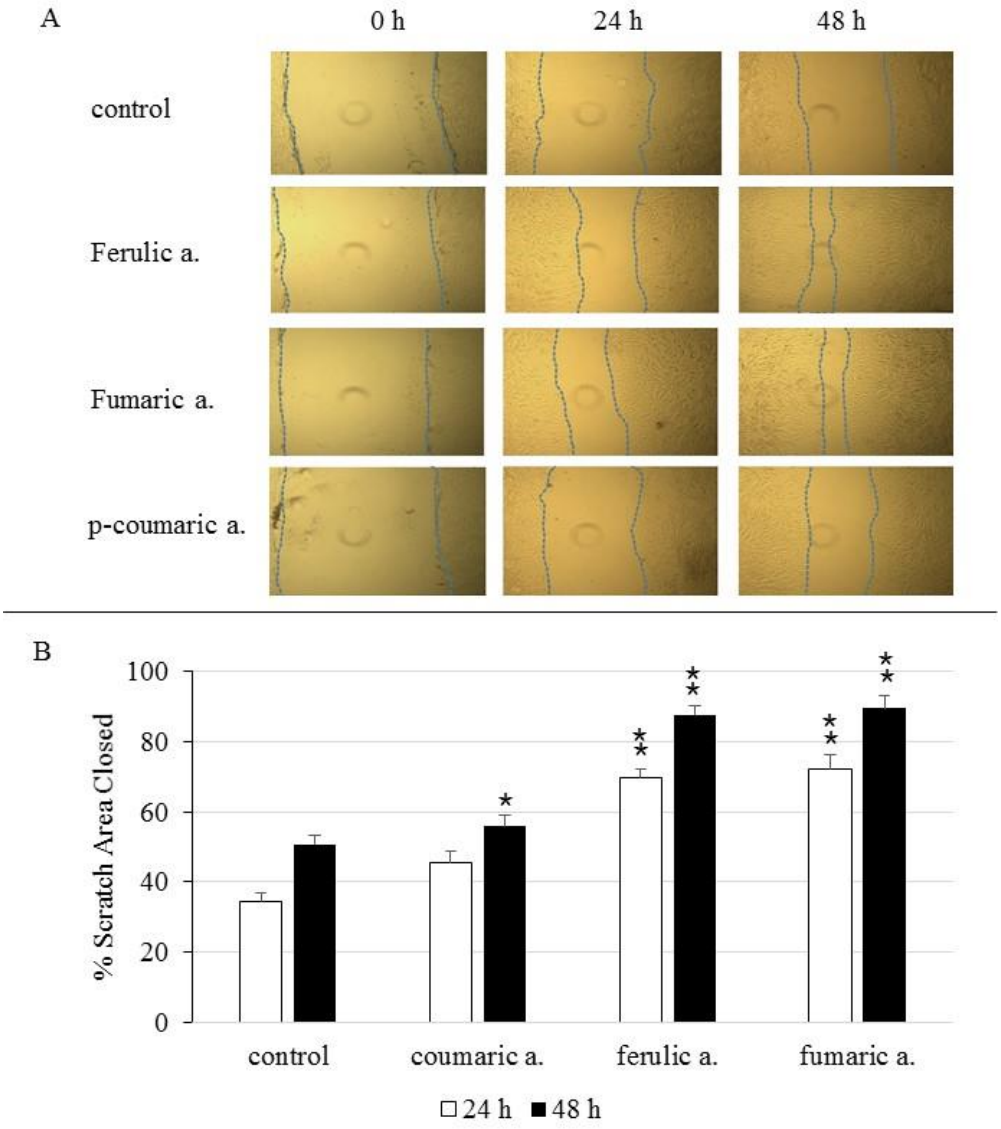

Figure 3. Wound closure effect of ferulic acid, fumaric acid, and *p*-coumaric acid.
Light microscope images of wound closure using fibroblast cells. **A**: The images show the cell migration into the scratched wound area in the presence of 100 µM of chemicals and the control. Images were captured at 0, 24, and 48 h and compared with controls. **B**: Scratch closure was analyzed with the measurement of the empty scratched area. Each time point was compared to the image on day 0 and reported as a percentage of closure. Data are presented as the mean±SD of 3 repeats per group. *$p < 0.05$ and **$p < 0.001$ compared with the untreated control.

It has been known that fumaric acid is an intermediate product in the citric acid cycle of many organisms, which is a source of intracellular energy in the form of adenosine triphosphate. Several studies have described anti-oxidative, anti-inflammatory, and immunomodulatory properties of fumaric acid [42]. Fumaric acid esters as a medicine have been used in the treatment of psoriasis. In a clinical study, psoriasis plaques were treated with fumaric acid and were shown to induce glutathione and Nrf2 pathway genes in the skin plaques of patients with psoriasis [43-44]. In biopsy materials, the transcription factors PTTG1, NR3C1, GATA3, and NFkBIZ were specifically regulated, which are important in normal skin development, and the T-helper and Th17 pathways [45]. Besides, fumaric acid was also used in wound dressing materials to improve wound healing performance [46]. The researchers believe that it could further enhance the healing performance of wound dressing materials by the anti-oxidative and anti-inflammatory effects. However, exact mechanisms of synergistic effects are not yet sufficiently explained. We report here for the first time that fumaric acid, which is also found in LS, possess effective wound healing properties by increasing the proliferation and migration of skin fibroblasts. Further study is necessary to identify the exact mechanism of this effect.

Trans-ferulic acid, *p*-coumaric acid and caffeic acid identified in LS by our study are phenolic molecules, which are also found in many foods, such as vegetables, fruits, and some plants. They have potent antioxidant, anti-cancer, antimutagenic, anti-inflammatory, anti-microbial, and anti-neurodegenerative and immunomodulatory properties, both in vitro and in vivo [47-49]. As related to wound healing, it was shown that ferulic acid effectively enhanced the wound healing process in diabetic rats by increasing the collagen content and re-epithelialization [50, 51]. Another work also showed that ferulic acid stimulates the cellular proliferation and migration of human oral fibroblasts [52]. The associated study proposed that ferulic acid can regulate cell mobility by controlling microtubule dynamics. Also, we here demonstrated that ferulic acid stimulated cellular proliferation and migration of dermal fibroblasts, although not as much as that of fumaric acid. Furthermore, our study showed the lower efficacy of *p*-coumaric acid as compared to fumaric and ferulic acid in the wound healing process in vitro. However, there is lack of sufficient evidence for the effects of *p*-coumaric acid on fibroblasts, although it has been known that it inhibits NF-kB signaling pathways, a transcription that regulates many genes that are involved in inflammation, immunity, cell survival, neural plasticity, and neurogenesis [53]. On the other hand, there is few literatures that caffeic acid enhances wound healing in vitro and in vivo [54-55]. As related to in vitro, it was demonstrated that caffeic acid significantly stimulated collagen-like polymer synthesis in NIH 3T3 fibroblast cells, suggesting that it appears to be effective on wound healing [56]. However, further research at the molecular level and more knowledge are required regarding the effect mechanisms of major components in LS on fibroblast before conclusions can be made.

In conclusion, this in vitro study demonstrates that *Lucilia sericata* larval secretion induce differential expression of some genes associated with wound healing in primary rat dermal fibroblasts have an enhancing effect on the migration of fibroblasts. Gene expression analysis showed that LS treatment modulated the expression of Col14a1, Cxcl11, Ctsl1, Fgf7, Hgf, Il10, Itgb6, Tnf, and Fgf2 transcripts of the fibroblasts during a period of 72 h incubation. The profile of differentially expressed genes with larval secretion treatment was mostly similar to those of several other medicines, which contribute to the enhanced healing of wounds in previous studies. In addition to waxy hydrocarbons identified by GC-MS analysis, the LC-HRMS analysis revealed the presence of fumaric acid, *trans*-ferulic acid, and *p*-coumaric acid as the major bioactive metabolites in LS and are responsible for the wound healing activity of LS. Fumaric acid was major component tested in LS and possesses significant wound healing potential by enhancing the migration abilities of the fibroblasts.

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Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

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