Peer-reviewed version available at *J. Clin. Med.* **2019**, *9*, 59; doi:10.3390/jcm9010059

Article

Ulmus Parvifolia Accelerate Skin Wound Healing by Regulating the Expression of MMPs and TGF-β

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Abstract: Ulmus species have been widely used in Korean folk medicine because of their anti-inflammatory and antimicrobial properties. We intended to investigate the wound healing effect of the powder of Ulmus parvifolia (UP) root bark in a mouse wound healing model. We also determined the mechanisms of effects of Ulmus parvifolia (UP) in skin and skin wound healing effect using keratinocyte model. in vivo experiments showed that the wound lesions in the mice decreased by U. parvifolia with 200 mesh size of root bark powder and significantly reduced by treatment with UP, compared with those treated with U. macrocarpa (UM). Results from in vitro experiments also revealed that UP extract promoted the migration of human skin keratinocytes. UP powder treatment upregulated the expression of the matrix metalloproteinase-2 and -9 protein and significantly increased transforming growth factor (TGF)-β levels. We confirmed that topical administration of the bark powder of exerted a significant effect on skin wound healing by upregulating the expression of MMP and transforming growth factor-β. TGF-β In, Our study suggests that U. parvifolia may be a potential candidate for skin wound healing including epidermal skin rejuvernation.

Keywords: Ulmus parvifolia, wound healing, matrix metalloproteinase, transforming growth factor, skin rejuvernation

1. Introduction

The Skin is composed of the dermis and epidermis layers and acts as a protective shield against environmental factors such as harmful UV rays and pathogens. It also prevents water loss [1]. Wound healing is a complex multistep overlapping process in which blood clot formation and wound inflammation are following by skin tissue proliferation and remodeling. Wounds begin to heal immediately after an injury due to the release of various clotting factors, platelet-derived factors (PDGF), and transforming growth factors. During the inflammatory phase, neutrophils and macrophages are activating by the release of pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, and TNFα and growth factors such as PDGF, TGF-α, TGF-β, IGF-1, and FGF. In the proliferative phase, these factors stimulate the activation, proliferation, and migration to the injured site of fibroblasts, keratinocytes, and endothelial cells, which then stimulates angiogenesis and extracellular matrix (ECM) formation. Finally, during the remodeling phase, fibroblast and vascular density

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decrease, old collagen fibers of the initial scar are replacing with matrix, and new collagen fibers are synthesizing to form new tissue [2-5]. Namely, skin wounds are caused by infections of numerous inflammatory factors in addition to simple skin injury. Because the skin healing process is very complex, there can be limits to fully overcome skin wound injury with a single compound. Thus, the development of wound healing agents with natural products may be an option for cutaneous wound treatment. The use of the natural product as wound healing materials can have some advantage such as low cost and high safety in comparison to other synthetic agents.

For thousands of years, many examples of applications on skin using natural resources have been reported. Even though, in order to actually apply it clinically, further precisely scientific studies are needed. Actually, over the last 10 years, many papers have been reported evidences that natural products can improve skin wounds. [6]. As part of such research, this study was conducted to demonstrate the pharmacological function of elm tree for skin wounds.

The elm tree is widely distributed in Asia, and its stem and root barks, etc have used in traditional oriental medicine to treat gastric disorders and intestinal inflammation [7]. Pariticularly, it has long been used in the regenerating stomach or skin epithelial cells in Korea Elms are also known for their effects on blood circulation, the protection of cartilage degeneration, and damaged tissue regeneration [8]. With regard to topical use, elms have been administered for the treatment of minor skin irritations, cold sores, ulcers, abscesses, and boils [9]. Elm trees are found in the Ulmus genus in the Ulmaceae family, and the bioactivities of various Ulmus species have also been reported. Ulmus davidiana var. japonica reportedly has anti-oxidant, anti-inflammatory, and immune-modulating effects [10]. Recent studies on U. parvifolia Jacq. (UP), a species of elm native to China, Korea, and Japan, have shown that its leaves and stems have anti-inflammatory and antioxidant effects [11]. U. parvifolia bark, which contains phenolic compounds and steroidal glucosides, is used for the treatment of eczema and edema [12]. Water soluble extracts of the root bark of U. parvifolia reportedly showed anti-inflammatory properties, and co-treatment with the mycelia of mushroom protected against allergic asthma in mice [13,14]. Also, interestingly, it has been reported that U. parvifolia used the powder of the original material itself rather than being used only as an extract in the clinical purpose. Therefore, this study aimed to check the possibility of U. parvifolia as a candidate for skin wound healing including anti-inflammation. Firstly, we investigated the influence of dorsal treatment of U. parvifolia in the animal model of cutaneous wounds according to the particle size of the U. parvifolia root bark power. Continually, we further performed that comparative study of species differences such as U. parvifolia, and U. macrocarpa on potential efficacy in skin wound models.

2. Materials and Methods

2.1 Sample preparation

Ulmus parvifolia (UP) was collected from Busan and Jinju, provided from Prof. MJ Ahn at Gyeongsang National University, Jinju 52828, Korea, in April 2018. The root barks were washing with water, dried, and pulverized using a grinder. Each powder was sieved through 20, 50, 100, and 200 mesh sieves (pore sizes: 0.85, 0.35, 0.15, and 0.075 mm, respectively), to obtain 4 grades of root bark powder. UP powder was extracted twice in 80% methanol for 24h with 1h sonication. The solution was filtered through Whatman No.1 filter paper, concentrated using a rotary vacuum evaporator under reduced pressure

2.2 Measurement of the angle of repose of the powder

The angle of repose (θ) for the root bark powder was measured using the cone height method. Briefly, a funnel was fixed at a height of 30 cm (H) above ground level, and different sizes of the powder were allowed to gently flow through it until the tip of the powder cone touched the outlet

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of the funnel. The diameter (2R) of the cone was measured for each powder type. The angle of repose (θ) was calculated as follows:

$$\theta = \tan\text{-}1\ (h/r)$$

This test was performed in triplicate for each sample.

2.3 Wound-healing model

Specific pathogen-free 5-week-old male SKH-1 hairless mice were purchased (Orient Bio; Gyeonggi-do, Korea) and acclimatized for 1 week in a temperature- and humidity-controlled room (23 °C and 60% humidity), under a 12-hour light-dark cycle, before the start of the experiments. All experimental protocol for animal experiments was reviewing and approved by the animal care committee of the Center of Animal Care and Use (CACU, LCDI-2018-0007) at the Lee Gil Ya Cancer and Diabetes Institute, Gachon University, Korea. The mice were randomly divided into 2 sets and each set into 5 groups. Set A: (1) vehicle (n = 7); (2) 50 mesh (root bark powder of UP, 12 mg/kg n = 6); (3) 100 mesh (12 mg/kg, n = 7); (4) 200 mesh (12 mg/kg, n = 7); and (5) Madecassol® (Dongkook Co.; Korea, 12 mg/kg; n = 7). The part of two wounds was created in the posterior dorsal area of each mouse using a 6 mm biopsy punch, and the powders were topically applied to the wounds each day for 10 days. Set B: (1) vehicle (n = 8); (2) UP 200 mesh (20 mg/kg, n = 8); (3) U. macrocarpa (UM) 200 mesh (20 mg/kg, n = 8); (4) Madecassol® (20 mg/kg, n = 8); and. A 20-mm circular skin wound results in scar formation on the back of each mouse, and each powder was topically treating to the wound each day for 21 days.

2.4 Wound analysis and histological assessment

Digital photographs of the wounds were captured on each day of treatment or at day 0, 3, 7, 10, and 14, using a digital camera (Olympus), and ImageJ software (version 1.5a; Bethesda, MD, USA) was used measure the wound sizes. Mice were sacrificed at the end of experiments after grafting for histological assessment. The harvested wound areas, including a border of normal tissue, were immediately fixed in 10% neutral-buffered formalin. The specimens were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) and Masson's trichrome. ImageJ software (version 1.5a) was used for the quantification of collagen in tissue sections.

2.5 Western blotting

The harvested skin tissues were homogenized in Pro-prep solution (iNtRON Biotechnology; Seoul, Korea), and its lysates were centrifuged at 12000 ×g for 30 minutes. The proteins were separated by SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, MA, USA). The membranes were blocked with 5% nonfat milk for 2 hours and washed with TBS containing 0.05% Tween-20 (TBST) buffer. The membranes were incubated with primary antibodies of MMP-1, -2, -9, and TGF-β (Santa Cruz, USA), at 4 °C overnight. The blots were incubated with an HRP-conjugated secondary antibody for 1 hour. Immunoreactive bands were visualized with the Pierce ECL Western blotting substrate (Thermo Scientific; Rockford, IL, USA), using ChemiDoc (BioRad Laboratories, CA. USA)

2.6 Cell culture

HaCaT cells were obtained from the Korean Cell Line Bank (Seoul, Korea). The cells were cultured in high-glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in 5% CO2 at 37 °C.

2.7 Cell viability assay

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The cytotoxicity of UP extract was examined using the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HaCaT cells were seeded into 96-well plate (4.0×104 cells/well) in 10% FBS-containing medium. After 24 hours of incubation, 0.5 mg/mL MTT solution was added and the cells were cultured for 1 hour. The dark blue formazan crystals were solubilized with dimethyl sulfoxide (DMSO) and the absorbance at 570 nm was measured using a spectrophotometer (Molecular Devices, CA, USA).

2.8 Cell migration assay

HaCaT cells were seeded in 96-well plates (3.0 × 104 cells/well) for the scratching assay. Monolayers of cultured cells were subjected to scratch wounds with a Wound Maker tool (Essen Bioscience, MI, USA) and the media was removed by suction. The cells were then washed twice with PBS buffer and incubated for 12 hours in the presence or absence of UP extract. IncuCyte ZOOM (Essen Bioscience) was used to inspect cultures every 2 hours.

2.9 Statistical analysis

Differences between groups were determined using a one-way analysis of variance (ANOVA). p-values of <0.05, <0.01, and <0.001 were considered statistically significant. Results are presented as the mean 6 the standard error of the mean (SEM).

3. Results

3.1. The angle of repose of different particle sizes of the root bark powder of U. parvifolia

The angle of repose indicates changes in the fluidity in the root bark of UP. The angle of repose for the different particle sizes of the root bark powder of UP are shown in Table 1. The root bark powder of UP with a particle size of 200 mesh (49.8 ± 1.1°) had a lower angle of repose than the others, followed by 100 mesh (51.9 ± 1.6°) and 50 mesh (52.9 ± 0.7°), with the highest being the 20 mesh (57.2 ± 0.8°). The angle of repose of the root bark powder decreased as the particle size decreased

Table 1. The angle of repose of U. parvifolia root bark powder depends on particle size

Mesh	Particle size (μm)	Angle of repose (θ)
20	355–850	57.2 ± 0.8
50	150	52.9 ± 0.7
100	75–150	51.9 ± 1.6
200	≤75	49.8 ± 1.1

3.2 Effect of the particle size of the root bark powder of U. parvifolia on wound healing in mice

We observed the regenerative effects of the root bark powder of UP using a SKH-1 hairless mice model. To assess the efficacy of UP powder, wound closure was observed after treatment with UP powder (50, 100, and 200 mesh) for 5 days. Wounds treated with the 200 mesh size powder showed a faster rate of wound closure and dermal regeneration compared with those treated with other sizes (Figure 1A). In the 200 mesh size treatment group, wound sizes were significantly decreased on day 5, whereas those in the control group were not healed (Figure 1B). In addition, we investigated the tissue samples of skin wounds using H&E and Masson's trichrome (MT) staining

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(Figure 1C). Treatment with UP powder resulted in increased granulation tissue formation and collagen deposition in the 200 mesh size treatment group compared with other treatment groups.

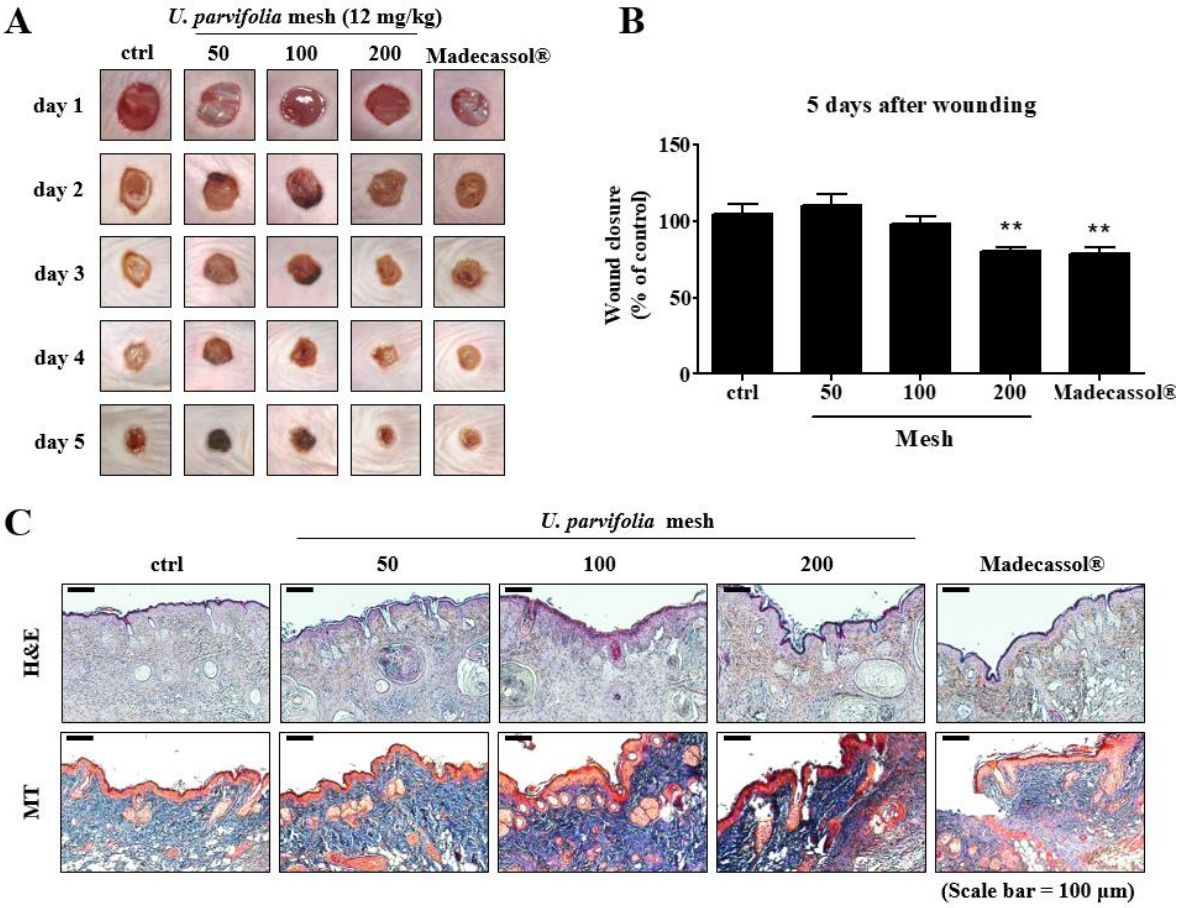

Figure 1. Effects of the root bark of U. parvifolia on wound healing in hairless mice. (A) Representative images of wounds from each group over a 5-day period post-wounding. Madecassol® was used as positive control. (B) The graphical representation of the average wound area in each group was measured using ImageJ software. (C) H&E-stained skin tissue sections and Masson's trichrome-stained sections on day 5. Scale bar = 100 μm. The values are shown as mean ± SEM (n = 7). **p <0.01 vs. the control group.

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3.3 Effects of root bark extract of U. parvifolia on migration in HaCaT cells

To determine whether root bark extract of U. parvifolia affected rejuvenation and wound repair, we induced wounds in skin keratinocyte (HaCaT cells) monolayer cultures and administered root bark extract of U. parvifolia . The cytotoxicity assay showed that 10 μg/ml of root bark extract of U. parvifolia had no cytotoxic effects (Figure 2A). HaCaT cells grown in the presence of root bark extract of U. parvifolia showed faster, dose-dependent growth rates compared with the untreated cells (Figure 2B-C).

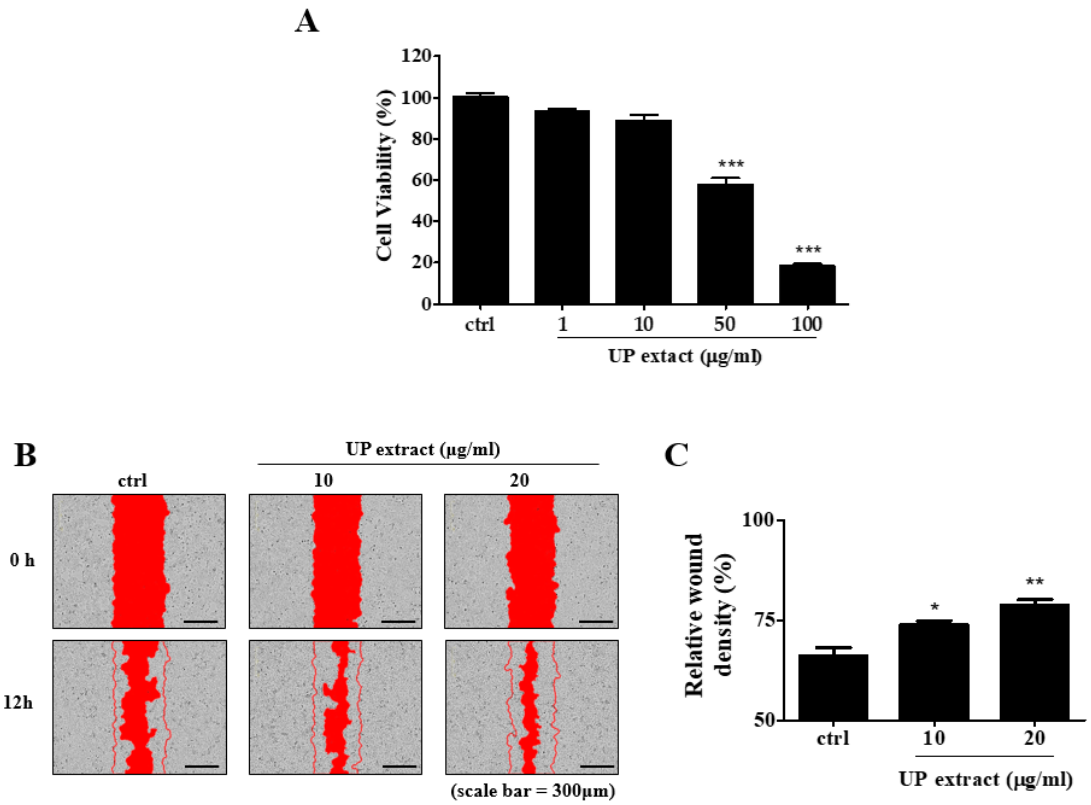

Figure 2. Effects of UP extract on migration in HaCaT cells. (A) Cells were cultured in 96-well plates and treated with UP extract (1, 10, 50,and 100 ug/ml). After 24 hours, cell viability was measured using the MTT assay. (B, C) Relative wound densities were recorded over time using the IncuCyte ZOOM™ live-cell imaging platform. HaCaT cells were cultured with or without UP extract . The red line indicates the initial scratch wound mask, created immediately after wound creation. The values are shown as mean ± SD (n = 6). *p <0.1 and **p <0.01 vs. the control group.

3.4 Effect of the root bark powder of U. parvifolia on large scale wound healing in mice

To investigate the effect of the root bark powder (200 mesh) of UP in large scale wound healing, we observed its regenerative effects using a 20-mm diameter wound created on SKH-1 mice. We also treated the wound with the root bark powders of UM to compare the effects of these with that of UP powder. Wounds treated with UP powder showed a faster rate of wound closure and dermal regeneration, similar to treatment with Madecassol® powder, 7 and 14-day post wound creation 3A). Seven and fourteen-day post wound creation, the wound sizes in the UP-treated group were significantly decreased, whereas those in the control and UM-treated groups were not significantly different as they were not completely healed (Figure 3B-C). Masson's trichrome staining was done to investigate wound development in tissue samples of wounded skin. UP

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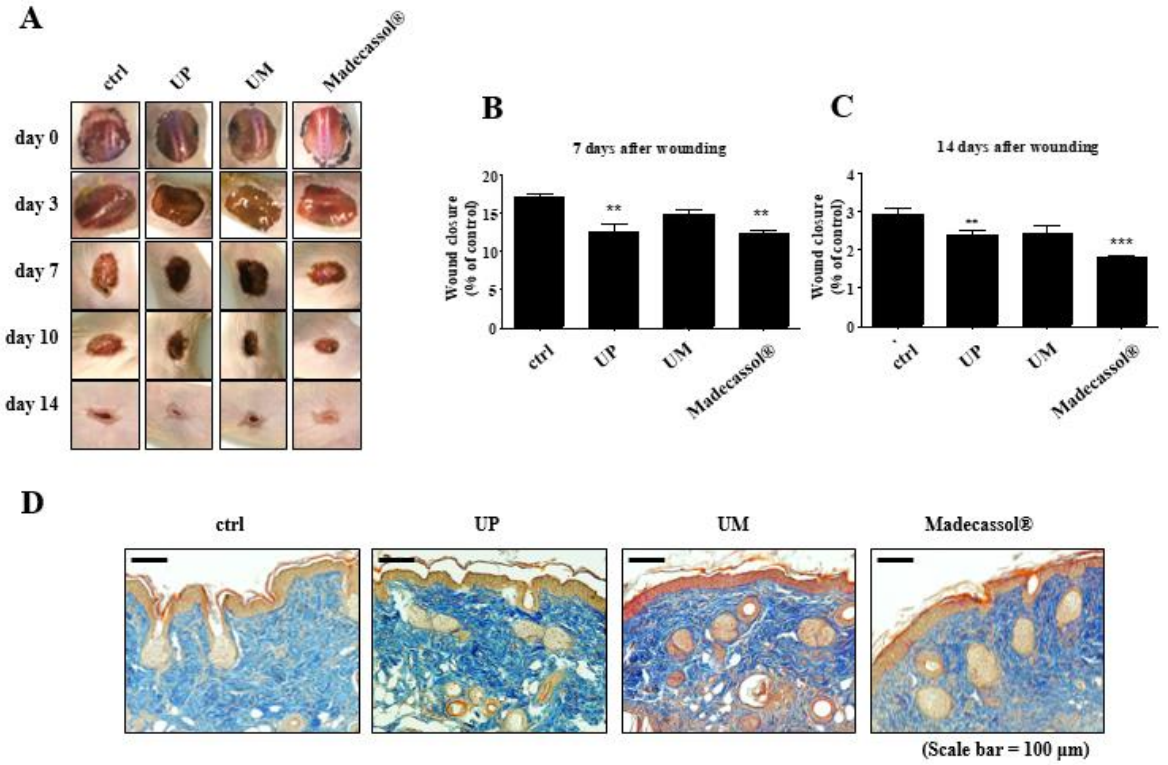
powder treatment resulted in more granulation tissue formation and collagen deposition than other treatments (Figure 3D). These results indicate that UP accelerate skin wound healing by enhancing collagen synthesis during the remodeling phase of the wound healing process

Figure 3. Effects of the root bark of U. parvifolia, U. macrocarpa on wound healing in hairless mice. (A)



Two Ulmus root barks were applied to the wounds of SKH-1 mice for 14 days. Madecassol® was used as positive control. (B and C) The closure rates of 20-mm diameter wounds were measured on day 7 and 14. (D) Masson's trichrome-stained tissue sections on day 14. Scale bar = 100 μm. The values are shown as mean ± SEM (n = 7). *p <0.05, **p <0.01, and ***p <0.001 vs. the control group.

3.5 Effect of U. parvifolia on skin wound healing in hairless mice by regulating MMP and TGF-β expression

We explored the expression levels of MMP-1, -2, -9, and TGF-β in the mice on day 14th of UP treatment (Figure 4A). UP treatment significant decreased the protein expression of MMP-1 (Figure 4B). On the contrary, the expression of MMP-2 or -9 was upregulated in the UP-treated group compared with the Madecassol®-treated group (Figure 4C-D). TGF-β levels were also increased in the UP-treated groups (Figure 4E). These results indicate that UP accelerate wound healing by enhancing the expression of MMP-2 and -9 and increasing TGF-β levels.

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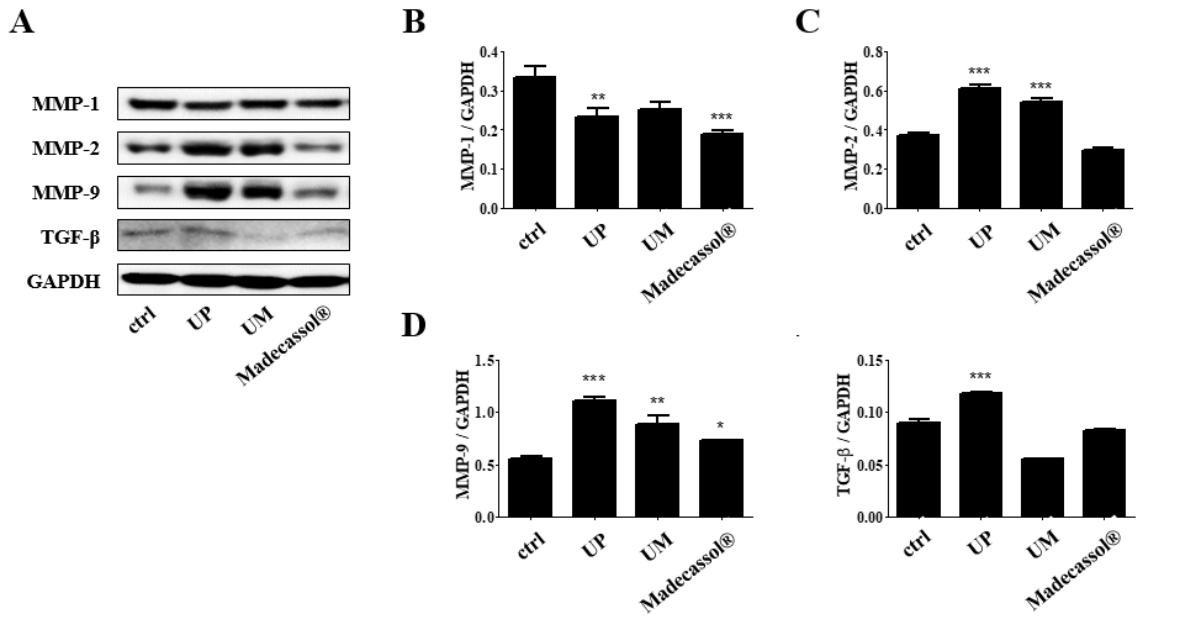

Figure 4. Effects of the root bark of U. parvifolia on the expression of MMPs and TGF-β in mice dorsal skin tissue. (A) Western blot analyses of wounded skin showed the expression of (B) MMP-1, (C) MMP-2, (D) MMP-9, and (E) TGF-β on day 14 post-wounding. The values are shown as mean ± SEM (n = 7). *p <0.05, **p <0.01, and ***p <0.001 vs. the control group.

4. Discussion

Ulmus species have been widely used in Korean traditional medicine because of their anti-inflammatory and antimicrobial properties. The bioactive components such as sesquiterpenoids, triterpenoids, flavonoids, coumarins, and lignans are mainly present in this species [15]. It has been reported that U. parvifolia have analgesic and anti-inflammatory effects [11,13]; however, its role in skin wound healing has not been reported. Therefore, in the present study, we have demonstrated for the first time the skin wound healing effect of the root bark of U. parvifolia in SKH-1 hairless mice.

Wound healing is a multistep overlapping process, which includes an inflammation, proliferation, and remodeling phase. Tissue re-epithelization and keratinocyte migration are essential processes in wound healing [16]. As population over 60 years of age grows, burdens of nonhealing cutaneous wounds such as pressure ulcer and diabetic foot ulcers are increasing [17]. The cutaneous wounds are particularly hard to heal in aging, so it is necessary to develop effective treatments to recover wound in aged skin. Treatment with powder in wound area absorbs more wound exudate, formed a crust that prevented overdrying, seals the wound from bacteria, can modulate maintenance moisture balance in the wound bed, and also reduce the lingering of malodor compared to ointment application. [18]. In the present study, it was observed that the wound closure and dermal regeneration effects of the 200 mesh size root bark powder of UP are similar to those of Madecassol®, a commercially available wound healing ointment [19]. When the angle of repose and size of particle were smaller, the solubility and water retaining the capacity of powder [20] were increased. It seemed that the smaller the particle size, the larger the surface area and the better the absorption of exudate from the wound. Interestingly, UP powder itself in addition UP extract, has been used for many years in Korean traditional medicine. And it is possible that insoluble fibers in UP powder itself may quickly absorb inflammatory exudate more than when presented in the form of a UP extract.

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Maintaining of hemostasis of collagen in the skin is very import issue in skin rejuvenation and integrity to the wound matrix. It is also essential for re-epithelization, cell-cell, and cell-matrix interactions. Deposition of collagen is important in wound healing and the development of wound strength [21]. The remodeling of collagenous proteins during wound healing can be influenced by proteolytic activities in the extracellular matrix by the matrix metalloproteinase (MMPs). In our study, treatment of UP sample with different particle size showed that the finest UP powder (200 mesh)- treated animals significantly decreased wound size and also increased the collagen level in the dorsal skin. Conclusively, UP powder with smaller particle size has stronger collagen maturation in wound healing process significantly.

During normal tissue remodeling and morphogenesis, MMPs play a crucial role in all stages of wound healing by modifying the wound matrix [22]. Understanding the role of MMPs during infection and chronic tissue repair may pave the way in identifying potential targets for chronic wounds. Also, MMPs regulate cell-cell and cell-matrix signaling through the release of cytokines and growth factors sequestered in the ECM. Previously, it was shown that cytokines and hormones modulate MMP expression in skin tissues and may regulate inflammation and ECM on skin tissues [23]. In our study, expression of MMP-1 was downregulated by treatment of UP similar to the positive control group. The loss of ECM may trigger MMP-1 expression in basal keratinocytes thereby promoting migration, but keratinocytes downregulate the expression of MMP-1 in the final stage of tissue remodeling [24]. Furthermore, UP treatment upregulated MMP-2 and -9 expressions even more than those of Madecassol® treatment. Particularly, over expressions of matrix metalloproteinases 2 and 9 impairs the remodeling and re-epithelization phases in wound damaged models. [25]. Downregulation of MMP-2 and -9 expressions in the wounds increased keratinocyte migration during wound closure. MMP-9 knockout mice delay wound re-epithelialization and inhibit cell proliferation through Smad2 signaling in delaying corneal wound healing [26,27]. Therefore, the potential of MMPs and their inhibitors will be as therapeutic agents in treating wounds during distinct phases of the wound healing.

Keratinocyte and fibroblast migration during the re-epithelization phase are important processes in mammalian skin healing. Keratinocytes are the predominant cell in the epidermis and responsible for the epithelialization phase of skin wound healing. During the epithelialization, keratinocytes proliferate and migrate to the wound sited. These processes help ameliorate the disruption of the skin barrier [28]. Impaired keratinocyte migration results in poor wound healing, leading to a chronic wound [29]. Therefore, regulation of keratinocytes migration by UP treatment may ameliorate wound lesion via regulating expression of MMPs.

TGF-β is a family of growth factors that play an essential role in wound healing by regulating the inflammatory response, keratinocyte proliferation and migration, angiogenesis, collagen synthesis, and ECM remodeling. Lower TGF-β expression were studied in skin of human diabetic foot ulcer [30]. Our results suggest that the potential efficacy of wound healing by UP seems to be due to stimulation the keratinocytes migration directly or TGF-β expression in wound lesion.

5. Conclusion

we for the first time discovered that the root bark powder of U. parvifolia accelerate wound healing, and that the mechanism might involve the upregulation of the expression of MMPs and TGF-β. Therefore, root bark powder of U. parvifolia can be a potential candidate in treating cutaneous wound damages. The further precise mechanism study on UP in skin cells and the effects of its main compound should be done.

Author Contributions: conceptualization, S.Y.K. and M.A.; methodology, M.C.K., W.S.P.; data curation, M.C.K., S.Y.; investigation, H.M.S.; writing—original draft preparation, M.C.K. and S.Y.; writing—review and editing, S.Y.K., K.H.K. and M.C.S.; visualization, M.C.K.; supervision, S.Y.K.; project administration, M.A.

Acknowledgments: This work was supported by the R&D Program for Forest Science Technology (Project No. 2017036A00-1719-BA01) developed by the Korea Forest Service (Korea Forestry Promotion Institute).

Conflicts of Interest: The authors declare that they have no conflicts of interest to declare.

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32. Title of Site. Available online: URL (accessed on Day Month Year).