Gynura procumbens

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Gynura procumbens, a herb found in Southeast Asia, may be an effective therapeutic for nonhealing diabetic wounds.

angiogenesis G. procumbens skin traditional medicine wound healing

1. Introduction

Nonhealing chronic wounds do not progress through the healing process in a timely manner and have become a major socioeconomic challenge to healthcare systems worldwide. To date, the prevalence rate for chronic nonhealing wounds in industrialized countries is approximately 2% of the general population, similar to the prevalence rate for heart failure [1]. Chronic wounds include but are not limited to diabetic foot ulcers, venous leg ulcers, and pressure ulcers [2][3]. Although various therapeutic strategies have been pro-posed to treat chronic nonhealing wounds, including debridement, offloading, endovascular treatment, surgery to promote revascularization and the use of growth factors to promote wound healing, these wounds heal slowly and can worsen rapidly [4][5]. A recent study reported that chronic nonhealing wounds impact nearly 15% of Medicare beneficiaries (8.2 million), demonstrating the economic impact and burden of chronic nonhealing wounds in the Medicare population [1]. There is, therefore, a substantially unmet need to develop new interventions that are effective and inexpensive.

Gynura procumbens (Lour.) Merr. is a small plant approximately 1–3 m in height with a fleshy stem and ovate-elliptical or lanceolate shaped leaves. It is a perennial plant that belongs to the Asteraceae family and is commonly found in tropical Asian countries such as China, Thailand, Indonesia, Malaysia and Vietnam. Leaf extracts of *G. procumbens* are used in folk medicine to treat various ailments, such as fever, inflammation, migraines, rheumatism, cancer, viral infections and hypertension [6]. In addition, the extracts of *G. procumbens* leaves exhibit antihyperglycemic, antihyperlipidemic and hypoglycemic properties in diabetic rats [7] and promote wound healing in healthy rats [8]. The wound healing process consists of hemostasis, inflammation, proliferation and remodeling [9][10]. This process involves several cell types, including keratinocytes, fibroblasts, endothelial cells and mast cells [11]. Moreover, various growth factors, such as angiogenin (ANG), epidermal growth factor (EGF), fibroblast growth factor (FGF), transforming growth factor (TGF)-β, platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), have been shown to facilitate the wound healing process [11].

Although *G. procumbens* has antiglycemic and wound healing properties, no report has been published on its effects on chronic nonhealing wounds or its ability to activate cells that play key roles in the wound healing

process. Our main purposes were to evaluate the effects of an ethanolic extract of *G. procumbens* leaves on wound healing in normal and diabetic mice and to investigate its stimulatory properties in endothelial cells, fibroblasts, keratinocytes and mast cells.

2. Chemical Constituents of G. procumbens as Determined by TLC and Phytochemical Screening

We first examined the components of the ethanolic extract of *G. procumbens* as determined by TLC with detection under UV light at 254 nm and 366 nm and using a natural product spraying reagent and anisaldehyde-sulfuric acid spraying reagent. The TLC chromatograms revealed kaempferol and quercetin and stigmasterol as chemical constituents of *G. procumbens* extract at Rf = 0.66, 0.38 and 0.32, respectively. The other standards, namely, chlorogenic acid, caffeic acid and rosmarinic acid, were not found in the composition of our ethanolic *G. procumbens* extract (data not shown). A previous study reported that chlorogenic acid is a component of an ethanolic extract of *G. procumbens* collected from HERBagus Sdn Bhd, Kepala Batas, Malaysia [12]. The discrepancy of this prior result and our study result may be due to differences in the geographical, climatic and/or experimental conditions. Moreover, a phytochemical screening of the ethanolic extract of *G. procumbens* revealed positive tests for phenolics, tannins, flavonoids, terpenes and other proteins.

3. Ethanolic G. procumbens Extract Promotes Wound Healing in Both Normal and Diabetic Mice

To examine the effect of the ethanolic extract of *G. procumbens* on in vivo wound healing, full-thickness wounds were created on the dorsal back of mice and 0.5% *G. procumbens* was topically applied.

Solcoseryl jelly (10%) was used as a positive control for the treatment of diabetic wounds [13][14]. Compared to that of the vehicle-treated normal mice, significant wound healing activity was first observed on day 2 in animals treated with the 0.5% *G. procumbens* extract. The *G. procumbens*-treated wounds were completely healed by day 16, while the vehicle-treated mice were completely healed by day 26. On the diabetic mice, the *G. procumbens*-treated wounds started to significantly heal on day 2 and complete healing was observed on day 22, which differed from the vehicle-treated group, in which complete healing was observed on day 35. Overall, *G. procumbens* shortened the wound healing time in healthy and diabetic mice by 30% and 40%, respectively. In both the normal and diabetic groups, *G. procumbens* accelerated wound healing in the mice more rapidly than solcoseryl jelly. Compared to the effect of solcoseryl jelly, *G. procumbens* rapidly accelerated wound closure from day 6 to day 16 in both healthy and diabetic mice. *G. procumbens* treatment caused neither toxicity nor mortality during or for at least 6 months after treatment (data not shown). All mice were healthy in growth, appearance and behavior.

4. G. procumbens Induces the Expression of Various Angiogenic Factors

Angiogenesis is critical for wound repair and is regulated by an extensive variety of angiogenic growth factors from various cells involved in the wound healing process.

Because *G. procumbens* induced wound healing, we speculated that it can also stimulate angiogenic growth factors. Skin tissues at the wound area were collected on days 2, 6 and 12 posttreatment and examined for the gene expression of angiogenic growth factors by RT-PCR. *G. procumbens*-treated wound tissues of the normal control and diabetic mice displayed high expression of various angiogenic factors, including ANG, EGF, FGF, TGF-β and VEGF. No significant differences were found between *G. procumbens*- and solcoseryl-jelly-treated wounds with respect to the induction of angiogenic factors in the control mice. However, interestingly, *G. procumbens* markedly increased the expression of VEGF 2 days postinjury and tended to increase the levels of EGF ($p = 0.1318$) and FGF ($p = 0.2199$) 6 days postinjury in the diabetic mice compared with solcoseryl jelly.

The wound healing process involves the coordinated action of several types of cells, such as endothelial cells, fibroblasts, keratinocytes and mast cells, which produce a multitude of growth factors that are indispensable at each stage of the wound healing process [11]. To determine whether *G. procumbens* activated these cells to stimulate angiogenic factors under in vitro diabetic conditions, cells were treated with 38 mM glucose to mimic the diabetic milieu [15], and mannitol was used as an osmotic control for the high-glucose treatment [16]. We confirmed that glucose indeed attenuated the induction of angiogenic factors in keratinocytes, fibroblasts, endothelial cells and mast cells, but mannitol had no effect on cell activation. Interestingly, *G. procumbens* significantly enhanced the mRNA expression of ANG, FGF, PDGF and VEGF in keratinocytes and endothelial cells under both normal and diabetic conditions. The *G. procumbens* extract also markedly induced the expression of FGF and VEGF in fibroblasts and ANG and VEGF in mast cells. The observation that *G. procumbens* stimulates angiogenic factors in human endothelial cells, fibroblasts, keratinocytes and human mast cells under diabetic conditions suggests that this herbal extract may have been involved in the angiogenesis of the diabetic mice.

5. G. procumbens Promotes Vascular Formation in Normal and Diabetic Mice

Given that the *G. procumbens* extract increased the expression of angiogenic factors that are generally reduced in diabetic wounds [17], we hypothesized that this extract might encourage diabetic wound vascularity. Newly healed tissues collected on the 8th day of treatment were histologically examined. Hematoxylin and eosin staining showed an increased number of large vacuolar vessels in the *G. procumbens*-treated tissues from the normal mice. This increased neovascularization was confirmed by an increased number of CD31-positive cells compared to the vehicle-treated wounds. Interestingly, in the diabetic mice, the *G. procumbens*-treated wounds had a strikingly increased number of vessels and displayed more CD31-positive cells than either the vehicle- or solcoseryl-jelly-treated wounds.

Furthermore, macroscopic observations of repaired wounds on both the normal and diabetic mice clearly revealed vascular formation in the wound areas treated with *G. procumbens*. The vessels were remarkably increased in both

number and size in the wounds treated with *G. procumbens* compared to the vehicle- and solcoseryl-jelly-treated wounds.

6. G. procumbens Enhances Mast Cell Accumulation and Migration

Mast cells accumulate in healing skin wounds and influence multiple phases of the wound healing process [18]. Tissues collected at wound areas on the 8th day of treatment were stained with toluidine blue and revealed that the *G. procumbens*-treated wound sites in the normal control mice displayed a substantial number of accumulated mast cells compared with the solcoseryl-jelly- and vehicle-treated wounds. *G. procumbens* treatment also remarkably increased the number of mast cells in the diabetic mice.

The ability of *G. procumbens* to attract mast cells in the diabetic milieu was further confirmed by an in vitro chemotaxis assay using the LAD2 human mast cells cultured in high glucose. We observed that spontaneous mast cell migration was weakened under high-glucose conditions. Under both normal and diabetic conditions, *G. procumbens* dramatically induced mast cell migration. *G. procumbens*-induced cell migration resulted in an eightfold and tenfold increase under normal conditions and diabetic conditions.

7. G. procumbens Promotes the Proliferation of Keratinocytes, Fibroblasts and Endothelial Cells

Wound healing is a dynamic reparative process that proceeds through a sequence of steps, including the proliferation and migration of different types of cells, such as keratinocytes, fibroblasts and endothelial cells [3]. First, we cultured these cells under high-glucose conditions and used a BrdU incorporation assay to determine whether *G. procumbens* can promote the cell proliferation. Significantly increased proliferation of keratinocytes, fibroblasts and endothelial cells was observed when these cells were treated with *G. procumbens* under both normal and diabetic conditions. A twelvefold increase was observed for keratinocytes under both normal and high-glucose conditions, a twofold and threefold increase was observed for fibroblasts under normal and high-glucose conditions, respectively, and a twofold increase was found in the endothelial cells under both normal and high-glucose conditions.

8. G. procumbens Accelerates Wound Healing In Vitro

Next, a scratch assay was used to assess the ability of *G. procumbens* to accelerate wound healing in vitro in keratinocytes, fibroblasts and endothelial cells that were cultured under both normal and high-glucose conditions. Because *G. procumbens* induces cell proliferation, all in vitro wound healing experiments were conducted in the presence of mitomycin C, an inhibitor of cell proliferation, to exclude the effect of proliferation on wound healing. Compared to the vehicle- or high-glucose-treated cells, the *G. procumbens*-treated cells rapidly migrated and covered the wound area under both normal and high-glucose conditions. Quantification of the wound closure is

shown in the right panels. *G. procumbens* promoted wound healing in the presence of mitomycin C, implying that *G. procumbens*-mediated wound healing can be mainly attributed to cell migration. The finding that *G. procumbens* induces cell proliferation and migration confirms the in vivo observation that *G. procumbens* promotes wound healing in diabetic subjects.

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