In-vitro Wound Healing Properties of *Commiphora swynnertonii* Resinous Extracts

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SUMMARY

Wound healing is a complex multicellular process involving many cell types which include; inflammatory cells, endothelial cells, fibroblasts and keratinocytes. The process involves an orderly sequence of events with four overlapping phases namely; haemostasis, inflammatory, proliferation and remodeling phases. The process can be facilitated by the use of wound healing agents including herbal remedies from plants. In this study the main objective was to evaluate the *in vitro* wound healing activity of the resin obtained from *Commiphora swynnertonii (C.swynnertonii)*. First the NIH -3T3 cells viability were evaluated using (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl Tetrazolium Bromide (MTT) assay. Then the wound scratch assay model was used to evaluate cellular proliferation, closure of the wound and release of matrix metalloproteinase enzymes. Results indicate differences in mean cell viability between different concentrations within 24 hours of incubation. The highest viability was recorded at the concentration of 1% (v/v). The *in-vitro* wound scratch assay showed positive NIH - 3T3 cells proliferation on the wound area and cells migration when compared with control group (without treatment) at 0 and 24 hours. In addition, *C. swynnertonii* was able to stimulate secretion of MMP-2 release from NIH - 3T3 cells. MMP-2 is an important enzyme for extracellular matrix remodeling during wound healing suggesting that *C. swynnertonii* promotes wound healing by stimulating cell proliferation and production of MMP-2 in a mechanism that is currently not known.

Key words: Wound, *Comiphora*, healing, remodeling, metalloproteinases, NIH - 3T3 cells

INTRODUCTION

Skin is the protective barrier of the animal's body against the outside environment and thus, any break to it must be rapidly and efficiently repaired in a process known as wound healing. Wound healing is a complex multicellular process involving many cell types which include; inflammatory cells, endothelial cells, fibroblasts and keratinocytes. The process involves an orderly sequence of events with four overlapping phases namely; haemostasis, inflammatory, proliferation and remodeling phases. (Guo and DiPietro, 2010; Sultana *et al*., 2015). Various biomolecules are involved in the regulation of the wound healing process. These molecules include extracellular matrix (ECM), integrins, growth factors and matrix metalloproteinases (MMPs) (Krejner *et al*., 2016). Cellular migration on the ECM,

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remodeling and degradation of the ECM by MMPs are very important in wound repair. These phases have been documented to be fueled by several factors including the use of antibiotics and other wound healing agents from naturally occurring medicinal plants (Muniandy *et al.,* 2018). Many plants derived products have been traditionally used for the treatment of wounds in developing countries (Muniandy *et al.*, 2018). The choice of herbal products for the treatment of wounds and other ailments depends on the tradition and plant species found in different regions of the world. Recently, many efforts have been made worldwide to discover agents of plant origin that can promote wound healing, reduce cost of treatment and save patients from complications such as amputation (Oguntibeju, 2019). *Commiphora swynnertonii* is a tropical plant which has been used by most pastoralist communities for treatment of various diseases. Previous studies have demonstrated that the extracts form *C. swynnertonii* exhibit various biological activities such as antimicrobial effect (Bakari *et al.,* 2012; 2013), reduces blood sugar and cholesterol (Bakari *et al*., 2015 and Maghembe *et al*., 2017) and increases proliferation and synthesis of blood cells (Bakari *et al.,* 2017). Furthermore, resin from other *Commiphora spp* have shown to facilitate healing power on the surgical wounds (Muniandy *et al.,* 2018). The main objective of the present study was to evaluate *in-vitro* wound healing activity of the resin obtained from *C. swynnertonii*.

MATERIALS AND METHODS

Study plant and resin collection

The plant material (resin) used in this study was collected from the northern Tanzania District of Simanjiro in Manyara Region, which is located 4°0′0″S and 36°30′0″E. The district has an altitude of 1,360 m above sea level. The plant was first identified and verified by a botanist as *Commiphora swynnertonii* and a voucher specimen number CK 6489 was preserved at the National Herbarium in Arusha (J. Kayombo, personal communication, 2009). The resin of *C. swynnertonii* was collected into wide mouthed glass bottles and transported to Sokoine University of Agriculture for extraction and testing.

Preparation of the resinous extract

In the laboratory, the resin was soaked in ethanol (99.8 % v/v) then filtered twice through cotton wool placed on a funnel and then concentrated immediately using rotary evaporator. The resulting filtrate (crude resinous extract) was then stored at 4 °C in air tight amber bottles until use.

Cell viability (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl Tetrazolium Bromide MTT) assay.

This assay is used to assess Cell viability and was done following the method described by Lee *et al*., 2014. In this assay, rodent fibroblast cells (NIH - 3T3) were seeded in 96-well flat-bottomed tissue culture plates with 5 X 10^3 cells/well in 100 µL of the Dulbego cells modified medium (DMEM), and incubated at 37°C (5% CO_2 and 95% air) for 24 hrs. After 24 hrs the medium was flashed away and replaced by 10 µl of various concentrations (Negative control (1 % DMSO) / 0.01 / 0.1 / 0.2 / 0.5 / 1 / 5 / 10 / 20 / 50 / 100 volume %) of ethanoic resin extracts into the culture media in the plate. The plates were then incubated for another 24 hrs where 100 µL of MTT was added to each well and incubated again for 3 hrs. Then swirled gently and kept in a dark place at room temperature for about 30 mins. The ELISA microplate reader was used to read absorbance at 450nm. The absorbance readings in triplicate were used to calculate the NIH - 3T3 cells viability. The mean viability and standard error was calculated from triplicates (3 wells) for each respective *C. swynnertonii* concentrations and controls.

Wound scratch assay

The wound scratch assay was used to assess wound healing using a standardized procedure described by Liang *et al*. (2007) slightly modified by Chen (2012). The NIH - 3T3 were seeded in a 6–well culture plates at the concentration of 2 X 10^4 cells/well

cultured in DMEM with 10% foetal bovine serum (FBS) and grown as a monolayer to 70~80 % confluence. The medium was changed and replaced with various concentrations (Negative control (1 % DMSO) / 0.0001 / 0.01 / 1 %) of ethanoic extracts. The plates were then incubated at 37°C (5% CO_2) for 24 hours. Following incubation, a small area was then scratched gently and slowly across the center of the plate using a 1 mL pipette tip. The long-axial of the tip should always be perpendicular to the bottom of the well while scratching.

The resulting gap therefore will be equal to the outer diameter of the end of the tip. After scratching, the wells were gently washed twice with medium to remove the detached cells. Then the wells were replenished with fresh medium and incubated for another 24 hours. Then, the cells were washed twice with PBS, and then fixed with 3.7 % paraformaldehyde for 30 min. The gap between the two layers of the cells which were scratched was then inspected microscopically at 0, 24 and 48 hours. As the fibroblasts migrate to fill the scratched area, the images were captured with a digital camera attached to the microscope and computer system using image J software.

Matrix metalloproteinase inhibition using Gelatin zymography assay

MMPs are group of enzymes responsible for the degradation and remodeling of extracellular matrix during wound healing and cell migration thus aiding in wound closure and repair. The procedure was performed as explained by Park *et al*., (2018). The presence of MMPs on the granulation tissues of NIH - 3T3 tissue cultures was analyzed by gelatin zymography. One hundred milligrams (wet weight) of tissue were homogenized with Tris buffer (saline 0.9%, Tris 0.05 mg, Triton X-100 0.25%, and $CaCl_2$ 0.02 M) and centrifuged at 6000 rpm for 30 min. Tissue extract was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide containing 0.1% gelatin under nonreducing conditions without prior boiling. After electrophoresis, the gel was washed in 1.5% Triton X-100 for 1 h and subsequently immersed in buffer containing Tris-HCl 50 mM (pH 7.5), 1% Triton X-100, $CaCl_2$ 10 mM, and 0.02% sodium azide for 16 h at 37°C. The gel was washed several times with distilled water and stained with 0.25% Coomassie brilliant blue R250/40% methanol/10% acetic acid and destained in 7% acetic acid. Enzymatic activity was detected as clear bands of gelatin lysis against blue background. The molecular weight was determined by parallel running of samples in SDS-PAGE using standard protein markers (Genei Pvt. Ltd., Bangalore, India).

RESULTS

Cells treated with *C. swynnertonii* showed better viability compared to controls at lower concentrations up to 1% and at higher concentrations resulted into poor viability of cells (Figure 1A). Treatment with lower concentration of *C. swynnertonii* up to 1% stimulates proliferation and closure of the wound after 24hours (Figure 1B). There was no differences between 24hours (Figure 1B) and 48 hours (Data not shown) in terms of wound closure after scratch assay. Analysis of expression of matrix metalloproteinase enzymes using gelatin zymography showed strong expression of MMP-2 (55-75 kDa) in control treatments and weak concentration of *C. swynnertonii* compared to higher concentrations of *C. swynnertonii* (Figure 1C-D). Expression of MMP-9 (82-92 kDa) was very low (Data not shown).

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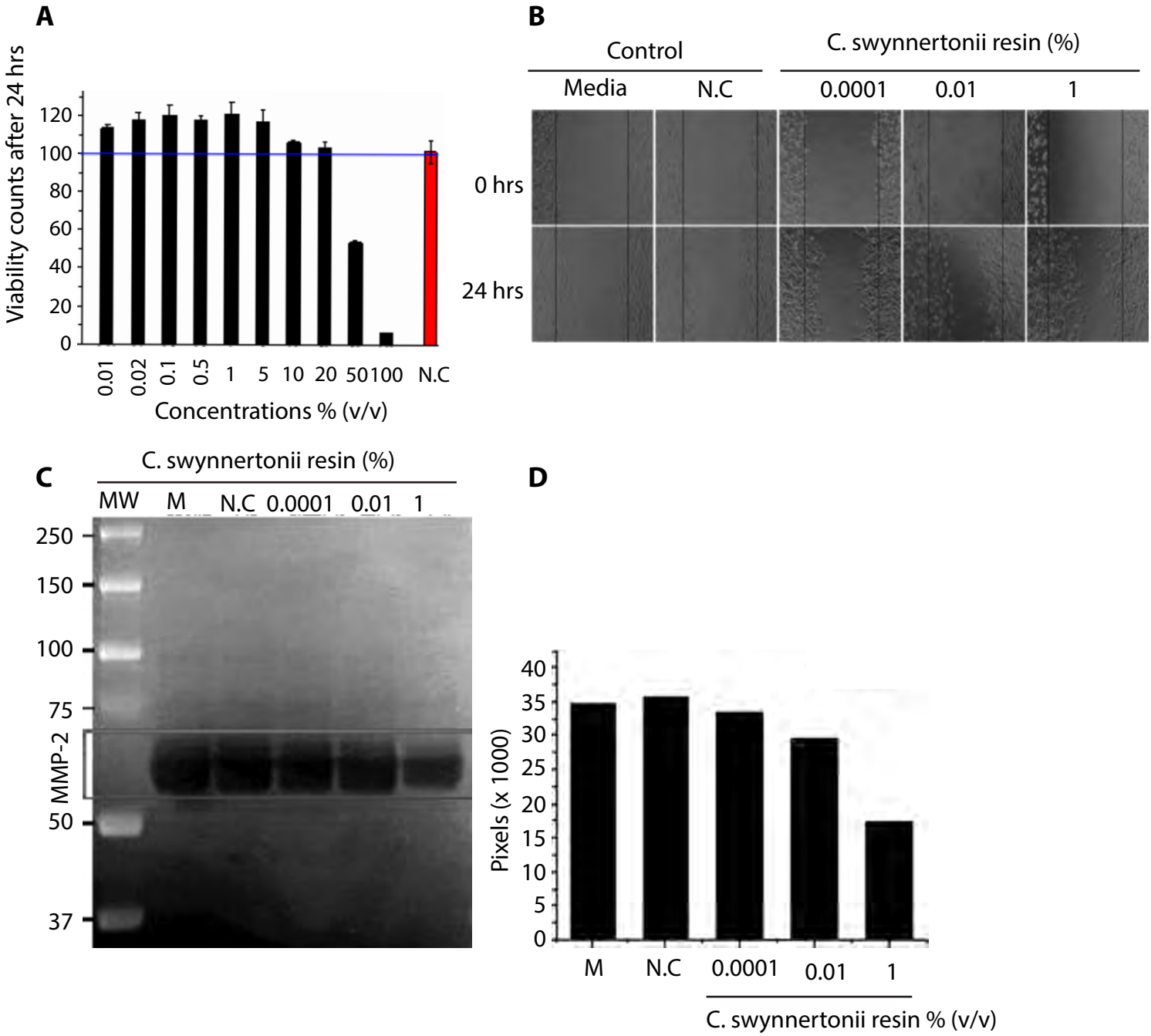

Figure 1. (**A**). Cell viability of Mouse fibroblasts (NIH - 3T3) cells at different concentrations of C. swynnertonii after 24 hours. The red bar (NC) is the mean viability for negative control (1% v/v DMSO), the black bars are the mean viability of NIH-3T3 cells after treatment with resins. The maximum cell viability is attained at 1 % v/v concentration and decreases as the concentration of the extract increases. Histograms represent mean viability in three wells, error bar represent standard error of the mean. (**B**) In vitro scratch assay (X 100 magnification). At the start of the experiment (upper panel) and end of the experiment (lower panel). Migration of the fibroblasts increases with increasing concentration of C. swynnertonii up to 1% (v/v) compared to Control (medium only) and NC (1% v/v DMSO). The gap (region between vertical lines) represent scrat area. (**C**) Gelatin Zymography of MMP 2 showing expression of MMP 2 (55 – 72 kDa) from NIH-3T3 cells. MW: Molecular weight marker in kDa, M: Medium only as control, N.C: Negative control (1% v/v DMSO). (**D**). Quantification of MMP-2 band intensity (pixels) for the respective treatment.

DISCUSSION

In the present study, the effect of *C. swynnertonii* resin methanolic extracts on wound healing was evaluated *in-vitro* using NIH - 3T3 cell lines and wound scratch assay. The wound scratch assay can be performed as a convenient, inexpensive and potential *in vitro* technique to evaluate wound healing properties of products using cell lines. The findings from this study revealed that, *C. swynnertonii* resin promotes fibroblasts survival following treatment with different concentrations (Figure 1A). These findings concur with the study done by Oguntibeju (2019) who reported different criteria for the product to qualify as wound healing agent including the ability to stimulate fibroblast proliferation among others. *C. swynnertonii* resin has also been reported to possess those characteristics (Bakari *et al*, 2012; 2017).

This is also supported by the observation on the wound scratch assay model in (Figure 1B**)** where increased fibroblasts proliferation was observed, with the ability to migrate into the scratched area within 24 hours of wound creation. However, there was no difference between 24 and 48hours incubation. Most likely this could be caused by the reduced proliferation ability after 24hours but actual mechanism remains a subject of further investigation.

During normal tissue remodeling and morphogenesis, MMPs plays the crucial role in all stages of wound healing by modifying the wound matrix (Caley *et al.*, 2015). MMPs regulate cell–cell and cell–matrix signaling through the release of cytokines and growth factors sequestered in the ECM. Down regulation of MMP-2 and -9 expressions in the wounds increased cell migration during wound closure (Gill and Park, 2008). The migrations help to ameliorate the disruption of the skin barrier via regulating expression of MMPs (Midwood *et al.,* 2004; Safferling *et al*., 2013). Thus the decrease in MMP expression after treatment with *C. swynnertonii* (Figure 1C-D) supports its wound healing properties through reducing the expression of MMPs to supports cellular migration and wound closure. However, further research is required to identify the mechanism how *C. swynnertonii* suppresses MMPs and whether *C. swynnertonii* directly stimulates cellular proliferation or proliferation occur indirectly through suppression of MMPs.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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