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RESEARCH ARTICLE

Wound-healing potential of methanolic extract of *Rhaphidophora korthalsii* leaves possibly mediated by collagen and fibronectin expression in L929 cell line

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ABSTRACT

Background: *Rhaphidophora korthalsii* is a climber shrub well known for its therapeutic properties in traditional medicine. The immunomodulatory properties of the plant extract have been investigated on mice splenocytes, human peripheral blood, and human peripheral blood mononuclear cell natural killer cells. The plant extract has also shown antioxidant effect and elevated macrophage production and has also been known to have anticancer properties. Yet, its wound-healing ability is not explored. **Aims and Objectives:** The objective of this research study is to evaluate the wound-healing potential of the plant extract on mouse fibroblast cell line L929 through scratch assay and the potential effect of the plant extract on the expression of two key wound-healing factors, fibronectin and collagen 1. **Materials and Methods:** L929 was exposed to various concentrations of *R. korthalsii* methanol extract to check the cytotoxic effect of the extract. *In vitro* scratch assay was performed on the treated cells to assess the wound-healing ability of the extract. Further, the effect of the extract on the expression of wound-healing factors, fibronectin and collagen 1, was studied through a flow cytometric analysis. **Results:** *R. korthalsii* leaf extract showed no toxicity on the L929 fibroblast cells. The treated cells showed ~93% wound closure after 48 h of incubation. The treatment of cells with the leaf extract, also, upregulated the expression of the wound-healing factors, fibronectin and collagen 1, i.e., 76.30% and 96.09% of the cells expressed them, respectively. **Conclusion:** *R. korthalsii* is a non-toxic plant with wound-healing properties. The phytochemicals in this plant may be responsible for its abilities. Thus, it can serve as an alternative therapy against synthetic drugs for wound healing.

KEY WORDS: *Rhaphidophora korthalsii*; Fibronectin; Collagen 1; Scratch Assay; Wound Healing

INTRODUCTION

Skin, a critical organ of the human body, forms the first line of defense in the immunological system.[1] Wounds rupture this immunological barrier making the system vulnerable to microbial infections and chemical agents. Chronic wounds, estimated to be prevalent in nearly 6 million people worldwide, are a major concern for both patients and clinicians.[2] Wound healing involves a complex process of tissue repair and remodeling involving a cascade of cellular and biochemical reactions.[3] These reactions ultimately reconstruct the extracellular matrix (ECM) through several processes, including fibroblast, keratinocyte, and macrophage migration, angiogenesis, and reepithelialization.[4-6]

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Connective tissue proteins such as collagen and fibronectin are critical to ECM reconstruction and form indispensable factors in wound-healing process.[5,7] Collagen and fibronectin synthesis and compaction by the fibroblasts orchestrate the wound-healing process by contracting the wound.[8-10] Therefore, discovery of wound-healing drugs involves the search for components that elevate the expression of collagen and fibronectin, thereby inducing the migration of fibroblasts and contracting the wound.

In this direction, the search for natural compounds that aid in wound-healing process has garnered the attention of the scientific fraternity. Although several medicinal properties of the plants such as antimicrobial,[11,12] antioxidant,[13,14] free radical scavenging,[15,16] anticancer,[17,18] and immunomodulatory[19,20] properties have been well studied, the search for plants that can be used as a source to isolate compounds that exert wound-healing effects has continued. Traditional and folk medicine practices from India and China have used wild plants and herbs to treat wounds, cuts, and burns.[2] However, their wound-healing effects need to be validated using well-established cellular and physiological models.

Rhaphidophora korthalsii, commonly known as "Dragon Tail" in Malaysia and Singapore, is a climber belonging to the *Araceae* family.[21] The plant is also distributed in other parts of South Asia including India, Sri Lanka, Indonesia, and Cambodia.[22] A decoction made of these plant leaves has been traditionally used in Indian and Chinese herbal medicine for treating cancer and skin ailments,[23] but scientific evidence that corroborates these traditional observations are limited. Earlier reports on *R. korthalsii* have identified its immunomodulating effect,[23] and the ability to stimulate the proliferation of mice splenocyte and human peripheral blood mononuclear cells *in vitro*.[23-25] However, the wound-healing potency of *R. korthalsii* remains to be studied.

In this study, we have addressed this lacuna by evaluating the wound-healing properties of the methanolic extract of *R. korthalsii* leaves by testing the migration of fibroblasts by *in vitro* scratch assay and the expression of ECM components – collagen and fibronectin.

MATERIALS AND METHODS

Reagents and Chemicals

Dulbecco's modified Eagle medium (DMEM) high glucose, fetal bovine serum (FBS), and Dulbecco's phosphate-buffered saline (D-PBS) used in cell culture and maintenance were purchased from HiMedia, India. Goat anti-collagen type I-fluorescein isothiocyanate (FITC) antibody (Southern Biotech, USA) and goat anti-fibronectin-FITC antibody (Novus Biologicals, USA) were used for the flow cytometric study. The human epidermal growth factor (hEGF) used as a positive control was from Roche, Switzerland. The cytotoxicity was measured by the 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay using MTT Reagent (HiMedia, India) and dimethyl sulfoxide (DMSO) (Sigma, USA). Cipladine was purchased from Cipla Ltd., India.

Collection of Plant Material

R. korthalsii was collected from the foothills of the Himalayas. Fresh plants were collected from Raisaar Devta, Tehri Garhwal District, Uttarakhand, India. Plant material [Figure 1] was identified by Dr. Kotresha, Assistant Professor in Department of Botany, Davangere University, Davangere, Karnataka.

Methanolic Extraction of Leaves

Methanol was used to extract secondary metabolites from plant leaves. The collected *R. korthalsii* leaves were chopped, air-dried, and minced by grinder. The leaf powder was mixed with methanol in the ratio 1:4 (w/v) and subjected to continuous stirring using a magnetic stirrer for 24 h. The plant extract was filtered through Whatman no. 1 filter paper. The extract was further concentrated and dried using a rotary evaporator at 40°C. Stock concentration of 1 mg/mL was prepared by dissolving in 50 µl of 99.99% DMSO and 950 µl of DMEM with 10% FBS and stored at room temperature.

Cell Culture

The mouse fibroblast cell line L929 was obtained from National Center for Cell Science, Pune, and cultured in DMEM with 4.5g/L of glucose premixed with 10% FBS and antibiotics (streptomycin 100 µg/mL and penicillin 100 U/mL) under 5% CO_2 at 37°C. After reaching 80% of density, the cells were trypsinized, and they were counted using a hemocytometer. Appropriate density of cells was seeded in T25 flasks and cultured until further usage of cells to conduct cell-based assays.

Cytotoxicity Assay

The cytotoxicity effect of *R. korthalsii* leaf extract was analyzed on L929 cells by performing MTT assay.[26] MTT is a tetrazolium salt, which is converted into insoluble purple-colored formazan crystals by the action of LDH enzyme released by the mitochondria.[27] L929 cells at a density of 2×10^4 were plated in a 96-well plate and cultured for overnight at 37°C and 5% CO_2. After attachment of cells to the surface of cell culture plate, the spent medium was replaced with DMEM having various working concentrations of plant extract (31.25, 62.5, 125, 250, and 500 µg/mL). Further, the L929 cells were incubated for 48 h at 37°C and 5% CO_2. After incubation, cells were treated with 20 µL of 5 mg/mL MTT reagent and incubated for 2 h at 37°C. The produced formazan crystals were dissolved with 100 µL DMSO, and the absorbance of purple color reagent was read at 570 nm in a microplate reader. The viability of cells treated with DMEM alone was considered as 100%, and the percentage of cell viability was calculated using the formula below:

% viability = ([OD of test sample treated cells − OD of blank] × 100)/(OD of untreated cells − OD of blank).

In Vitro Assessment of Cell Migration and Wound Closure

Scratch assay is a widely applied *in vitro* method to estimate the wound-healing ability of various therapeutic agents.[28] In the current study, 2×10^5 cells/mL of L929 cells were seeded in 6-well tissue culture plates for overnight at 37°C and 5% CO_2. The spent culture medium was removed, and a wound was created using a sterile 200 µL tip and the detached cells were washed with Dulbecco's phosphate-buffered saline (DPBS) twice. 125 µg/mL and 5 µg/mL of plant extract and positive controls, respectively, were added to respective wells and incubated for 48 h. The cell migration and wound closure were observed at different time intervals under an inverted microscope and images were taken by a digital camera. The gap filled due to migration of cells at different time intervals was measured using ImageJ software. The scratch assays were performed in triplicates (n=3).

Expression Studies by Flow Cytometry

The effect of *R. korthalsii* methanol extract on the most pivotal molecular factors of wound healing, fibronectin and collagen 1 expressions, was analyzed by flow cytometry. L929 cells at a density of 2×10^6 cells/mL were seeded in a 6-well microtiter plate and cultured overnight. Later, cells were exposed to 125 and 10 ng/mL of plant extract and positive control, hEGF, respectively, for 48 h. The cells were trypsinized and washed with DPBS. For intracellular collagen 1 expression studies, 70% ice-cold methanol was used to fix the cells by incubating the cells for overnight at −20°C. Cells were washed with DPBS and stained with anti-collagen 1 antibody and incubated under dark conditions at room temperature for 30 min.

Figure 1: *Rhaphidophora korthalsii* plant

For fibronectin expressions studies, the trypsinized cells were washed with DPBS twice and stained with antifibronectin antibody and incubated under dark conditions at room temperature for 30 min. Flow cytometric measurement of protein expressions was assessed by fluorescence-activated cell sorting Calibur, and the data were analyzed by CellQuest Pro software.

Statistical Analysis

All the experiments were conducted in triplicates, and the results were expressed as mean ± standard deviation (n=3). Statistical significance was determined by single-factor one-way analysis of variance for MTT assay and by paired t-test for scratch assay and expression studies; $P < 0.05$ was considered statistically significant. All statistical analyses were done in Microsoft Excel 2016.

RESULTS

In Vitro Cell Viability Assay

The cytotoxic effect of the methanolic extract of *R. korthalsii* plant leaves on mouse fibroblast L929 cell line was studied using the MTT assay. Table 1 and Figure 2 demonstrates the percent cell viability of L929 cells treated with various concentrations (31.25–500 µg/mL) of the extract for 48 h. The percentage viability of untreated cells was considered 100%. There was no significant decrease in the viable cell population across the treatment, suggesting that the extract had no cytotoxic effect on the cells. The percent viability of the cells at the highest concentration of 500 µg/mL was found to be 97.24 ± 0.13%, showing no cytotoxic effect.

In Vitro Assessment of Cell Migration and Wound Healing

Fibroblast cell migration is a critical indicator of wound healing. The wound-healing properties of the methanol extract of *R. korthalsii* leaves were assessed through *in vitro* scratch assay. There was a significant increase in the percentage of cell migration with time in the L929 cells that were treated with the methanolic extract of *R. korthalsii* leaves. The percentage wound closure at 0, 12, 24, and 48 h

Table 1: Cell viability of *Rhaphidophora korthalsii* extract treated L929 cell line

Culture conditions	% of cell viability
Vehicle control (µg/mL)	99.27±0.44
31.25	99.39±0.40
62.5	99.18±0.60
125	98.19±0.28
250	97.74±0.15
500	97.24±0.13

in L929 cells that were untreated [Figure 3a-d], treated with 125 µg/mL of *R. korthalsii* extract [Figure 3e-h], or treated with the 5 µg/mL of the standard drug Cipladine [Figure 3i-l] has been represented. The cells treated with 125 µg/mL of *R. korthalsii* extract migrated to a significantly greater extent when compared with the untreated cells. The quantified results have been represented as a graph of percent cell migration versus time in Figure 4.

Effect of *R. korthalsii* Treatment on Fibronectin Expression

Fibronectin is an adhesive molecule that plays an important role in wound healing processed, viz., extracellular matrix (ECM) formation and reepithelialization.[7] It interacts with various cells, cytokine molecules, and the ECM, thus aiding in wound closure.[8] Hence, the level of fibronectin expression in L929 cells before and after treatment with the extract was analyzed using flow cytometry. hEGF was used as the positive standard control. Fibronectin expression was found to be significantly higher in the extract-treated cells when compared to the untreated cells as represented in Figures 5 and 6. The percentage of extract (125 µg/mL)-treated cells that stained for fibronectin was 76.30% when 96.08% of the cells treated with positive control hEGF (10 ng/mL) expressed fibronectin.

Effect of *R. korthalsii* Treatment on Collagen 1 Expression

Collagens are the most abundant type of protein and can be found in all multicellular animals.[29] Their abundant functions include cell adhesion, tissue morphogenesis, cell migration, and tissue repair. Collagen type 1 is present in the dermis and is responsible for maintaining skin structure and cell integrity. It also plays a crucial role in wound healing.[5]

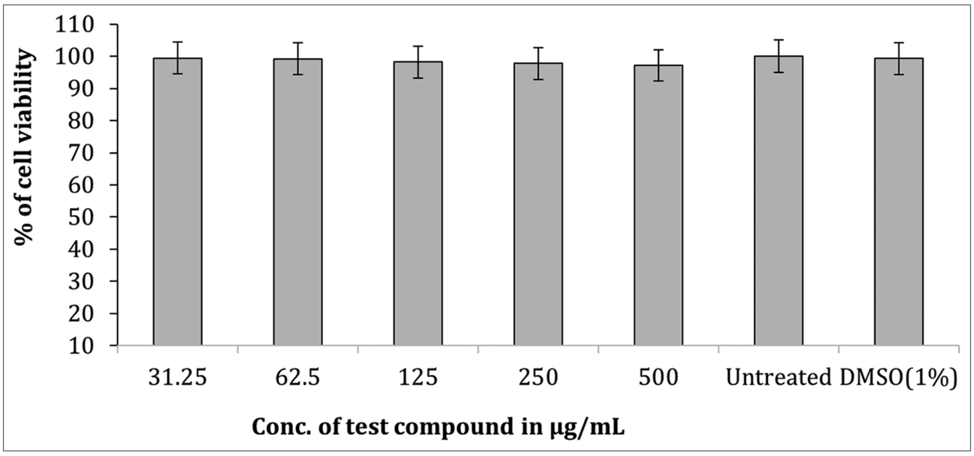

Figure 2: Effect of *Rhaphidophora korthalsii* methanol extract on mouse fibroblast cell line, L929. The graph represents the percentage cell viability on treatment with different concentrations of the extract for 48 h. The values are represented as mean ± standard of three experiments

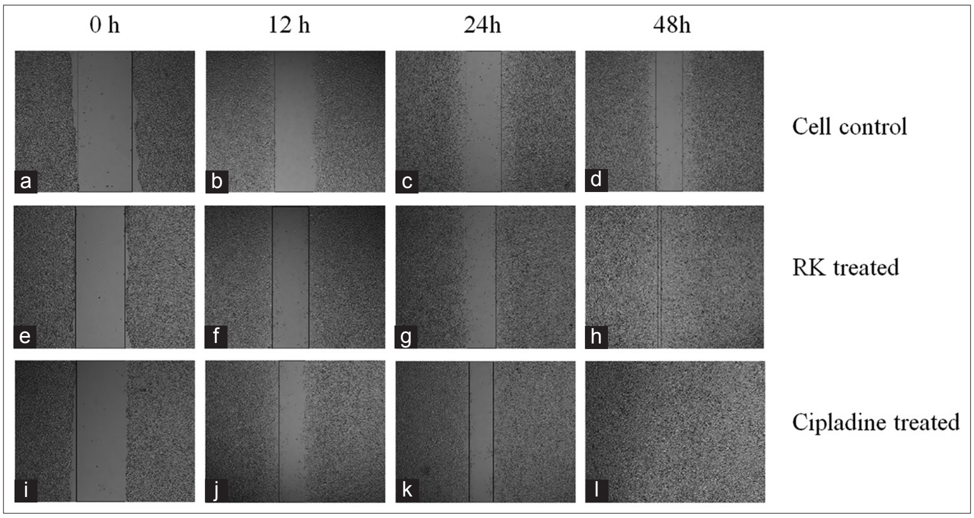

Figure 3: *In vitro* scratch assay. The images represent cell migration in untreated (a-d), *Rhaphidophora korthalsii* leaf extract treated (e-h) and the positive control drug cipladine treated (i-l) L929 cells at 0, 12, 24, and 48 h of treatment. For extract treatment, 125 µg of the extract was used. For positive control, 5 µg of cipladine was used for treated. The untreated cells were considered negative control (a-d). The boundaries of the scratched lines were determined by dark lines and the width the gap was measured using ImageJ software

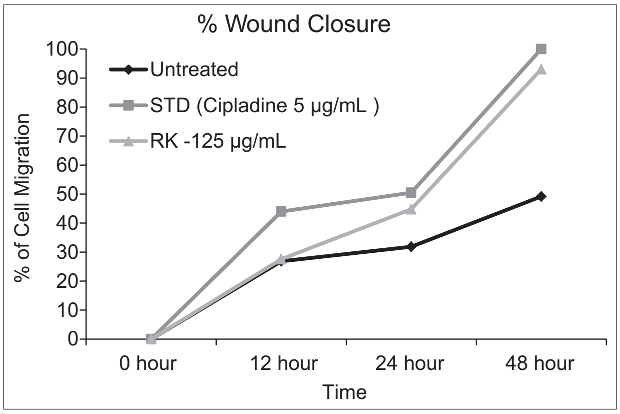

Figure 4: The effect of *Rhaphidophora korthalsii* leaves extract on migration of L929 fibroblast cells. The cells were treated with 125 µg/mL of the extract or 5 µg/mL of cipladine (standard drug). Blue: Cells with culture medium alone; Orange: 5 µg/mL of standard drug cipladine; Gray: 125 µg/mL of *R. korthalsii* leaf extract

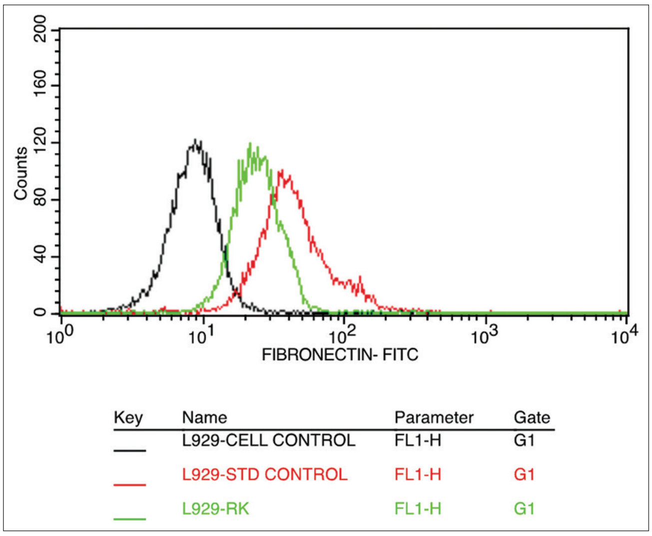

Figure 5: Overlaid expression of fibronectin in untreated L929 cells (black), positive control, human epidermal growth factor (10 ng/mL)-treated cells (red), and 125 µg/mL of *Rhaphidophora korthalsii* leaf extract-treated cells (green). The leaf extract-treated cells showed elevated expression compared to the untreated control cells

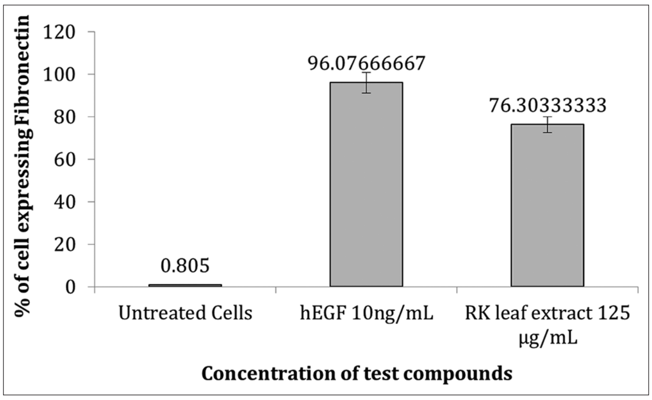

Figure 6: Fibronectin expression in untreated L929 cells, human epidermal growth factor (10 ng/mL)-treated cells, and 125 µg/mL of *Rhaphidophora korthalsii* leaf extract-treated cells after 48 h

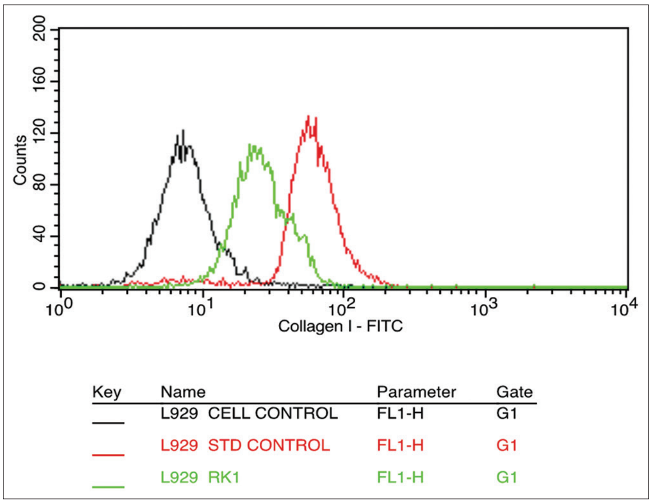

Figure 7: Overlaid expression of collagen 1 in untreated L929 cells (black), positive control, human epidermal growth factor (10 ng/mL)-treated cells (red), and 125 µg/mL of *Rhaphidophora korthalsii* leaf extract-treated cells (green). The leaf extract-treated cells showed elevated expression compared to the untreated control cells

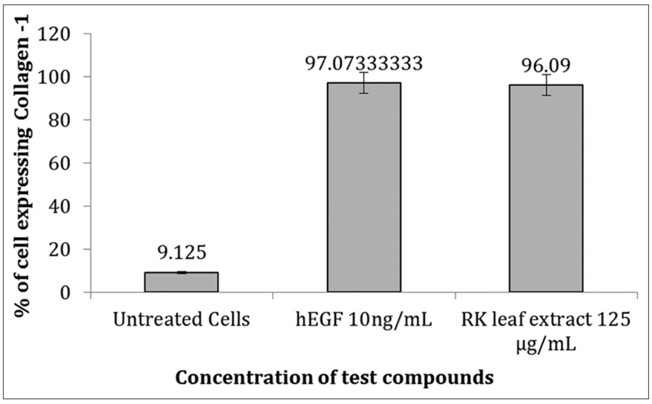

Figure 8: Collagen 1 expression in untreated L929 cells, human epidermal growth factor (10 ng/mL)-treated cells, and 125 µg/mL of *Rhaphidophora korthalsii* leaf extract-treated cells after 48 h

Collagen 1 expression in untreated, extract-treated, and control drug-treated L929 fibroblast cells was analyzed by flow cytometry. hEGF (10 ng/mL) was used as the positive standard control. L929 cells treated with the extract (125 µg/mL) for 48 h showed a significant increase in the collagen 1 expression and were similar to the expression in cells treated with the positive control as represented in Figures 7 and 8. Collagen 1 was expressed by 96.09% of the L929 cells treated with the leaf extract, much similar to the percentage of cells treated with the standard control, hEGF, that expressed collagen 1, i.e., 97.07%, suggesting the induction of collagen 1 expression in L929 cells by the methanolic extract of *R. korthalsii* leaves.

DISCUSSION

The cytotoxic effect of the methanolic extract of *R. korthalsii* plant leaves on mouse fibroblast L929 cell line was studied using the MTT assay. The percentage viability of untreated cells was considered 100%. There was no significant decrease in the viable cell population across the treatment, suggesting that the extract had no cytotoxic effect on the cells. The percent viability of the cells at the highest concentration of 500 µg/mL was found to be 97.24 ± 0.13%, showing no cytotoxic effect. Fibroblast cell migration is a critical indicator of wound healing. There was a significant increase in the percentage of cell migration with time in the L929 cells that were treated with the methanolic extract of *R. korthalsii* leaves. The cells treated with 125 µg/mL of *R. korthalsii* extract migrated to a significantly greater extent when compared with the untreated cells. Fibronectin expression was found to be significantly higher in the extract-treated cells when compared to the untreated cells. The percentage of extract (125 µg/mL)-treated cells that stained for fibronectin was 76.30% when 96.08% of the cells treated with positive control hEGF (10 ng/mL) expressed fibronectin. Collagen 1 expression in untreated, extract-treated, and control drug-treated L929 fibroblast cells was analyzed by flow cytometry. Collagen 1 was expressed by 96.09% of the L929 cells treated with the leaf extract, much similar to the percentage of cells treated with the standard control, hEGF, that expressed collagen 1, i.e., 97.07%, suggesting the induction of collagen 1 expression in L929 cells by the methanolic extract of *R. korthalsii* leaves.

Plants contain various phytoconstituents that can be employed in various therapeutic applications. Topical application of herbal concoctions on the site of injury and wounds has been a common practice in traditional medicine.[30] This wound-healing potential of herbal and plant extracts can be attributed to the antimicrobial, anti-inflammatory, antioxidant, and free-radical scavenging properties of phytochemicals present in them.[31,32] Some of these wound-healing properties of different plants have been tested in the past,[31-35] but the wound-healing properties of *R. korthalsii* – a plant well known for its other medicinal properties,[23-25] has remained unexplored. In this study, we evaluated the wound-healing properties of the methanolic extract of *R. korthalsii* leaves by *in vitro* scratch assay and expression studies on collagen 1 and fibronectin expression. The proliferative and migratory abilities of fibroblasts and keratinocytes are key features of the wound-healing process as previous reports have shown that fibroblasts migrate to the wound site and proliferate during the wound-healing process.[3,36] Stimulating the fibroblasts is one of the mechanisms by which the plant extracts might enhance the process of wound healing.[3] To understand the effect of the methanolic extract of *R. korthalsii* leaves in inducing fibroblast migration, we performed an *in vitro* scratch assay,[33] with a standard wound-healing drug, cipladine, as the positive control. Our results revealed that L929 cells migrated to a significantly greater extent when treated with the extract compared to the untreated cells, indicating that *R. korthalsii* leaves extract exhibit wound-healing abilities by inducing fibroblast migration. These data, therefore, suggest that the methanolic extract of *R. korthalsii* leaves closes the wounded area both by aiding in the migration of the fibroblast cells and their active proliferation at the wound site. Cellular changes during the process of wound healing are brought about by key molecular players such as collagen,[5,10] fibronectin,[7,8,10] platelet-derived growth factor-BB, and vascular endothelial growth factor. These molecules are pivotal to bringing about angiogenesis, reepithelialization, and ECM remodeling.[4,6,9] The synthesis of new ECM is brought about by elevated expression of connective tissue proteins such as collagen 1 and fibronectin. Fibronectin and fibrin interact to form the fibrin–fibronectin clot,[37] and later, during the inflammatory stage, fibronectin activates macrophages that phagocytose the cell debris, thus becoming a major component crucial for cell migration and wound healing.[7] Fibronectin also interacts with other wound-healing factors, especially, collagen.[34] Collagen type 1 constitutes 70% of the collagen present in the human body and plays a role in the last phase of wound healing, the tissue remodeling phase, where the ECM gets remodeled and the scar tissue is formed.[38] Therefore, an increase in the expression of fibronectin and collagen may be indicative of wound-healing properties of the test compound. For this reason, we studied the expression of collagen 1 and fibronectin in untreated, extract-treated, and control molecule (hEGF)-treated L929 cells. The experimental results revealed that the treatment of L929 cells with the extract significantly elevated the expression of collagen 1 and fibronectin. Further, the expression of collagen 1 in the extract treated cells was almost similar to the level of expression in the control-treated cells. These results suggest that the extract possesses wound-healing properties that bring about the migration of fibroblasts to the wound site and their proliferation at the site, orchestrated by the increased expression of collagen 1 and fibronectin. Further, these results corroborate previous reports that have shown an increase in the expression of fibronectin in wound-healing studies that involves plant phytochemicals. For instance, Sidhu *et al.* observed an increase in the expression of transforming growth factor-β and fibronectin in curcumin-treated wounds in animals.[35] To assess the safety of the plant material, we evaluated the cytotoxicity of the extract by MTT assay. The experimental results showed that the plant extract had no cytotoxic effect on L929 cells. The percent viability of the cells treated with the extract concentrations that were 16-fold higher than the half maximal inhibitory concentration value recommended as safe by most authors, and the American National Cancer Institute[39] were seen to have viability as good as 97.24%. Further, we found this result to be consistent with the previous report that showed the non-toxicity of methanolic extract of *R. korthalsii* on HepG2 cell line.[24] These results suggest that the extract has non-toxic effect on the cells and can be used for further studies.

This is the first of its kind of studies that established the wound healing potential of *R. korthalsii* leaves *in vitro*. Further studies were required to prove the wound-healing properties of this plant *in vivo* to understand the wound-healing potential of *R. korthalsii* leaves to extrapolate the beneficial effects of this plant to human beings.

CONCLUSION

Our study reveals the wound healing properties of the methanolic extract of *R. korthalsii* leaves brought about by the increased expression of collagen 1 and fibronectin and the induction of migration of fibroblasts to the wound site. Further, this extract has no cytotoxic effect and can be deemed as safe for further studies. These results establish the possible wound-healing properties of *R. korthalsii* and pave the way for further research to understand the phytoconstituents that bring about this effect.

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