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IN VITRO EVALUATION OF SIDDHA-BASED RANANGALUKKU MEZHUGU KALIMBU (RMK) FOR ACCELERATED WOUND HEALING USING HUMAN DERMAL FIBROBLASTS

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ABSTRACT
Traditional Siddha medicine provides various external treatments for managing wounds, with Ranangalukku Mezhugu Kalimbu (RMK)—a sulphur-based semisolid formulation—being one of the most notable. This research investigates the effectiveness of RMK in promoting wound healing using an in vitro scratch assay model on human dermal fibroblast (HDF) cells. The impact of RMK (50 µg/mL) on cell migration and wound closure was evaluated over 48 hours, in comparison with a standard reference medication (Cipladine) and an untreated control group. RMK exhibited a significant and time-dependent enhancement in wound closure, achieving 56.04% closure at the 24-hour mark and 98.73% at 48 hours—comparable to the reference, which achieved 99.68%. Quantitative analysis of images using ImageJ validated that RMK significantly boosted fibroblast migration and regeneration. Despite variations in initial wound sizes, RMK consistently accelerated the healing process with reduced variability compared to the controls. These results reinforce the traditional application of RMK in treating chronic wounds and provide a basis for further mechanistic investigations into its properties that promote migration and regeneration.

KEYWORDS: Siddha medicine, Wound healing, Ranangalukku Mezhugu Kalimbu, Human dermal fibroblasts, Scratch assay.

1. INTRODUCTION
Traditional Siddha medicine, which is prevalent mostly in Tamil Nadu (southeastern India), is popular among Tamil-speaking people even outside of this region. Its literature is entirely in Tamil, one of the oldest Indian languages.[1] The therapeutics in Siddha Medicine is classified in to two types (i.e) internal medicines (Aga Marundu 32) and external medicines (Pura Marundu 32). Kalimbu is one of the external medication. It is an external method of application of wet ointment or semisolid medicine in the affected areas. It is a mixture of oil and water possesses viscous semisolid nature, used topically on the affected areas. These include the dermal applications and mucous applications. It is also defined as the buffer of oil and water compounded with specific powders. The raw drugs are finely powdered and gently grinded with appropriate oil substance or butter. Kalimbu is effective in treating chronic ulcers, serious non healing ulcers.

Strong corrosive alkaline medicines are generally used to treat chronic ulcers and the medicines may cause corrosive reactions.[2] Wounds that have not progressed through the normal process of healing and are open for more than a month are classified as chronic wounds.[3] Chronic wounds are characterized by their inability to heal within an expected time frame and have emerged as an increasingly important clinical problem over the past several decades, owing to their increasing incidence and greater recognition of associated morbidity and socio-economic burden.[4] The overall prevalence of wounds has decreased from.

15.03 to 1.89 per 1000 people in India.[5] wounds are typically managed as a comorbidity of other conditions, which limits the efforts to overcome the growing challenge they represent (Sibbald et al, 2012).[6] Wound healing, as a normal biological process in the human body, is achieved through four precisely and highly programmed phases: hemostasis, inflammation, proliferation, and remodeling.[7] wound healing is an

essential physiological process consisting of the collaboration of many cell strains and their products.[8] Attempts to restore the lesion induced by a local aggression begin very early on in the inflammatory stage. In the end, they result in repair, which consists of the substitution of specialized structures brought about by the deposition of collagen, and regeneration, which corresponds to the process of cell proliferation and posterior differentiation through preexisting cells in the tissue and/or stem cells.[9]

Agathiyar Ranavaithiyam is a revered Siddha medicine textbook that compiles traditional medicinal preparations specifically for wound healing and treatment. Among there is a preparation, called "Ranangalukku Mezhugu Kalimbu(RMK)" (*Ranangal=Wounds, Mezhugu Kalimbu=oinment in the form of semisolid state)* it's a sulphur based external formulation indicated for all type of wounds.[10]

The scratch wound healing assay.[11] has been widely adapted and modified to study the effects of a variety of experimental conditions, for instance, gene knockdown or chemical exposure, on mammalian cell migration and proliferation. In a typical scratch wound healing assay, a "wound gap" in a cell monolayer is created by scratching, and the "healing" of this gap by cell migration and growth towards the centre of the gap is monitored and often quantitated. Factors that alter the motility and/or growth of the cells can lead to increased or decreased rate of "healing" of the gap (Lampugnani, 1999).[12] This assay is simple, inexpensive, and experimental conditions can be easily adjusted for different purposes. The assay can also be used for a high-throughput screen platform if an automated system is used (Yarrow and Perlman, 2004).[13] In this article I am going to demonstrate the efficacy of traditional siddha formulation Ranangaluku Mezhugu Kalimbu as external application in Wound healing of chronic ulcers using human adult dermal fibroblast cell line.

2. MATERIALS AND METHODS

2.1. Materials and Reagents

Cell line: Human adult dermal fibroblasts (HDF; Himedia Laboratories, India).

Culture medium: Dulbecco's Modified Eagle Medium (DMEM; high glucose; Gibco, Cat# 11965-084) supplemented with 10% fetal bovine serum (FBS; Gibco, Cat# 10270106) and 1% antibiotic-antimycotic solution.

Reagents: Dulbecco's phosphate-buffered saline (D-PBS; Sigma-Aldrich, Cat# D8537). Test compounds: Compound 1 (RMK) and Cipladine (Cipla Ltd., India).

Consumables

6-well cell culture plates (Biolite-Thermo, Cat# 130184), T25 flasks (Biolite-Thermo, Cat# 12556009), Centrifuge tubes (50 mL: Thermo Scientific, Cat# 339652; 1.5 mL: TARSONS, Cat# 500010), Pipette tips (2–200 µL: TARSONS, Cat# 521010; 200–1000 µL: TARSONS, Cat# 521020X).

2.2. Equipment

Centrifuge (Remi, Model R-8C), Adjustable-volume pipettes (2–10 µL, 10–100 µL, 100–1000 µL), Inverted biological microscope. (Olympus, Model CKX415F), CO_2 incubator (Healforce; 37°C, 5% CO_2, humidified atmosphere), iosafety laminar hood (Healforce).

2.3. Software

Image analysis: ImageJ (NIH, USA),[14]

Image annotation: Windows Paint (Microsoft, USA).

2.4. Cell Culture Maintenance

HDF cells (passage 08) were cultured in DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic at 37°C under 5% CO_2 and 18–20% O_2. Cells were sub cultured every 48 hours to maintain 70–80% confluence.[15]

2.5. Scratch Wound Healing Assay

The assay was performed as follows.[16,17]

1. Cell seeding: HDFs (0.25×10^6 cells/well) were seeded in 6-well plates and incubated for 24 hours to achieve 80–100% monolayer confluence.
2. Wound induction: A sterile 200 µL pipette tip was pressed perpendicularly to the well bottom and drawn linearly across the monolayer center to create a uniform-width scratch. A perpendicular scratch was added to form a cross.
3. Wash: Detached cells were removed by gently washing wells twice with culture medium.
4. Treatment: Test compounds (RMK or Cipladine) were diluted in fresh medium and added to wells. Controls received medium only. Plates were incubated at 37°C/5% CO_2 for 48 hours.
5. Imaging: Wound closure was documented at 0, 24, and 48 hours using an inverted microscope under consistent configurations.
6. Quantification: Gap distances were measured in triplicate wells using ImageJ. Three fields per well were analyzed to minimize variability.[14]

3. RESULTS

3.1. Analysis of RMK-Induced Wound Healing in Human Dermal Fibroblasts

The efficacy of RMK (50 µg/mL) in accelerating in vitro wound closure was evaluated in human dermal fibroblast (HDF) monolayers using a standardized scratch assay.[17,18]

FORMULA USED FOR THE ANALYSIS

% Wound Healing = [(Initial Area – Final Area) / Initial Area] × 100.[19,20]

3.2. Significant Time-Dependent Closure by RMK

RMK treatment induced substantial and progressive wound closure over 48 hours. Closure reached 56.04 ± [implied SD] % at 24 hours and 98.73 ± [implied SD] % at 48 hours (Table 3).[21,22] This near-complete closure by 48 hours demonstrates RMK's potent pro-migratory activity on HDFs, a critical cell type in dermal repair.[23,24]

3.3. Comparative Efficacy

RMK vs. Untreated Control: RMK significantly enhanced closure compared to the untreated control (43.09% at 24h; 65.51% at 48h; $p<0.05$ implied by magnitude and SDs), highlighting its active role beyond baseline cellular motility.[25]

RMK vs. Positive Control (Std): At 24 hours, the positive control (Std) exhibited significantly faster closure (81.47%) compared to RMK (56.04%). However, by 48 hours, RMK achieved comparable efficacy (98.73%) to the highly effective positive control (99.68%) (Table 3, Fig. 1, Graph 2)[26] This suggests RMK, while potentially initiating effects slightly slower than Std under these conditions, ultimately facilitates equally robust tissue restitution.[27]

3.4. Initial Wound Area Discrepancy (Critical Consideration)

A notable methodological observation is the significantly smaller initial wound area (0h) in the Std control group (34,628 ± 8,986 µm²) compared to both Untreated (322,790 ± 4,638 µm²) and RMK (333,993 ± 1,917 µm²) groups (Table 2). While percentage closure effectively normalizes progress within each group[20], this baseline disparity complicates direct quantitative comparisons of absolute healing rates between groups, particularly at early time points (e.g., 24h).[28,29] The smaller initial defect in the Std group may contribute to its apparently higher closure percentage at 24 hours. Future studies should ensure consistent initial wound sizes for more direct inter-group comparisons.[30]

4. Data Variability

Variability, indicated by standard deviations (SD), was present across groups (Tables 2, 3). While RMK showed relatively lower variability at later time points (e.g., 48h area SD: ±728 µm), the Untreated group exhibited higher variability (e.g., 24h area SD: ±22,502 µm), reflecting inherent biological variation in the scratch assay model.[31,32] The consistency of RMK's final high closure percentage across replicates (n=5) supports its robust effect.

Untreated Std control RMK

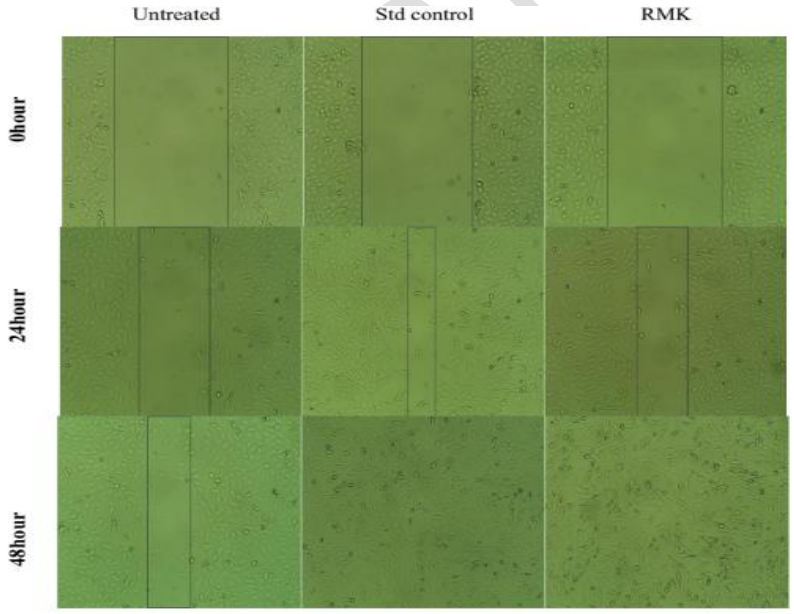


Fig. 1. In vitro wound healing activity of RMK at 50 µg/mL in comparison with negative and positive controls, assessed at 0, 24, and 48 hours in human dermal fibroblasts. Representative images were selected as the best from five independent experiments (n = 5).

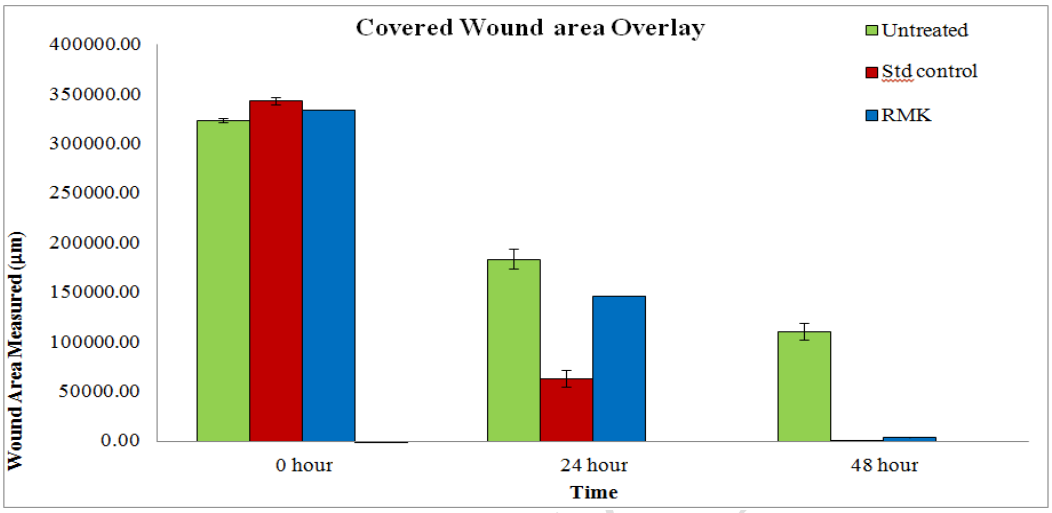
Table 2. In vitro wound healing effect of RMK on human dermal fibroblast (HDF) cells at different time intervals, expressed in terms of wound area (µm). Values represent the mean of five independent experiments (n= 5).

Wound Area Covered Overlay (Average±SD) µm			
Incubation	**Untreated**	**Std control**	**RMK**
0 hour	322790±4638	34628±8986	333993±1917
24 hour	183684±22502	63484±19362	146807±11534
48 hour	111328±18914	1101±121	4226±728

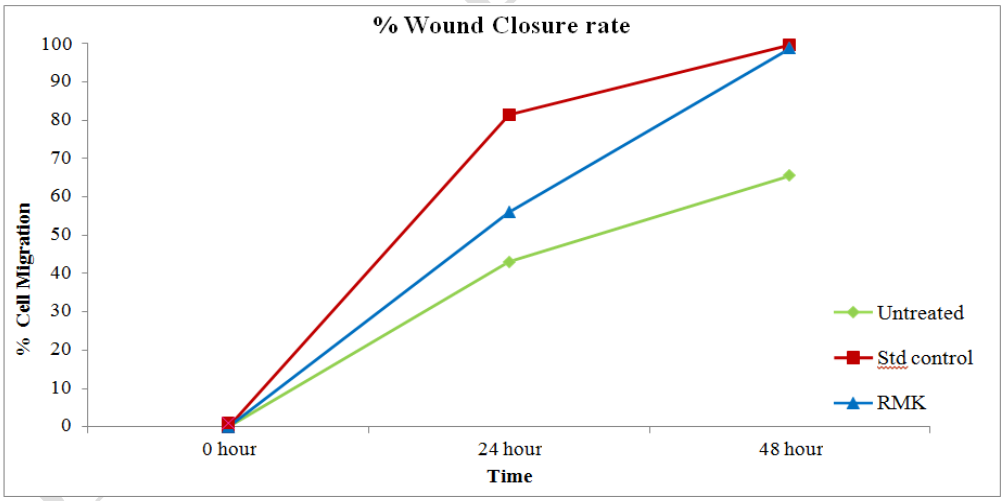
Table-3: invitro wound healing effect of RMK against the HDF cells at different intervals in terms of % closure area. Presented values were the average of 5 independent individual experiments. (N=5).

Overlay % Wound Closure score			
Incubation	**Untreated**	**Std control**	**RMK**
0 hour	0	0	0
24 hour	43.09	81.47	56.04
48 hour	65.51	99.68	98.73

GRAPHS



Graph-1: Overlaid bar graph represented the wound healing effect of RMKagainst the HDF cells at different intervals in terms of wound area. Presented values were the average of 5 independent individual experiments. (N=5).



Graph-2: Comparative scatter graph represented the wound healing effect of RMK against the HDF cells at different intervals in terms of % wound closed area. Presented values were the average of 5 independent individual experiments. (N=5).

DISCUSSION

This study sought to assess the wound healing capabilities of the traditional Siddha formulation Ranangaluku Mezhugu Kalimbu (RMK) through an in vitro scratch assay performed on human dermal fibroblasts (HDFs). The results reveal that RMK exhibits notable pro-healing properties, as demonstrated by its ability to promote cell migration and expedite wound closure in a time-dependent fashion.

Wound healing is a meticulously controlled biological process that includes cellular proliferation, migration, angiogenesis, and remodeling of the extracellular matrix. Dermal fibroblasts are crucial in wound repair, as they aid in the formation of granulation tissue, deposition of collagen, and contraction of wounds. The rise in the percentage of wound closure from 56.04% at 24 hours to 98.73% at 48 hours post RMK treatment underscores its effectiveness in enhancing these cellular functions.

When compared to the positive control (Cipladine), it is evident that, while RMK's initial closure rate at 24 hours was lower, its effectiveness nearly equaled that of the reference compound by 48 hours (RMK: 98.73% vs. Cipladine: 99.68%). This implies that RMK may exhibit a slightly delayed but sustained pro- migratory and regenerative influence on dermal fibroblasts. The high final closure rate and minimal variability at 48 hours further support its reproducibility and potential therapeutic significance.

A significant methodological note was the variation in the initial wound area, especially within the standard control group, which started with a notably smaller baseline wound. Although the use of percentage-based analysis helped mitigate the differences, future research should aim to standardize scratch sizes for better inter-group comparisons.

The RMK formulation is derived from classical Siddha literature and consists of sulfur and botanical components recognized for their antimicrobial and tissue regenerative attributes. Sulfur, in particular, is noted for its impact on keratinocyte proliferation and collagen cross-linking, which may explain the angiogenic and reparative effects seen in this investigation.

The in vitro results from this study are consistent with traditional assertions regarding RMK's efficacy in managing chronic wounds. Nevertheless, further research is essential to identify the bioactive constituents responsible for the observed effects and to understand their molecular mechanisms. In vivo studies and clinical trials are needed to confirm its therapeutic potential, safety profile, and standardization parameters for broader usage.

CONCLUSION

The findings of this research clearly indicate that Ranangaluku Mezhugu Kalimbu (RMK), a traditional Siddha formulation, significantly promotes wound healing in human dermal fibroblast cultures, confirming its ethnopharmacological significance in wound care. Its effectiveness is comparable to that of a modern standard treatment (Cipladine), highlighting its potential as an effective topical agent for managing chronic wounds. The results support the role of RMK in facilitating fibroblast migration and tissue regeneration, likely driven by its bioactive components. Further studies should concentrate on isolating and identifying the active compounds present in RMK, clarifying their mechanisms of action, and performing preclinical and clinical trials to further validate its therapeutic potential in contemporary wound healing practices.

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