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In vitro Wound Healing Effect of a Siddha Formulation: Gandhaga Thailam

R. Sathish Adithya [a,b++], E. M. Manikgantan [b#*], N. Kabilan [b†] and S. Kanimozhi [c#]

[a] Department of Nanju Maruthuvam, National Institute of Siddha, Chennai-47, India.
[b] Department of Siddha, Tamil Nadu Dr. MGR Medical University, Chennai-69, India.
[c] Sri Sairam Siddha Medical College and Research Centre, Chennai-44, India.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: Wound is defined as disruption in the integrity of skin. Wound healing is a physiological process primarily initiated by vasoconstriction and platelet aggregation. Wound closure is completed by release of inflammatory markers and aggregation of fibroblasts which causes reepitheliazation. Gandhaga thailam (Medicated oil), a siddha formulation prepared from Gandhagam (sulphur), Vediuppu (Potassium nitrate), Manosilai (Arsenic disulphide), Navacharam (Ammonium Chloride), and Veeram (Mercuric per chloride) triturated with cow ghee is used in a variety of skin diseases. Scientific validation of the wound healing efficacy of Gandhaga thailam remains unveiled.

[++] Assistant Professor, PhD Scholar;
[#] Associate Professor;
[†] Professor;
**Corresponding author: E-mail: manikgantan@gmail.com;*

Aim: The main aim of this research is to validate the wound healing efficacy of Gandhaga thailam through *in vitro* studies in L929 (Mouse fibroblast) cell line.
Materials and Methods: L929 (Mouse fibroblast) cell line was cultured in a 25 cm 2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate, and antibiotic solution containing: Penicillin (100 U/ml), Streptomycin (100 µg/ml), and Amphoteracin B (2.5 µg/ml). The scratch wounds were made by a sterile 1 mL pipette tip through a pre-marked line. The sample was checked for 0 hours, 24 hours, 48 hours, and 72 hours'.
Results: The results of the study shows that Gandhaga thailam is efficient in wound healing and the wound is closed by about 48th hour after the scratch. The faster wound healing efficacy of Gandhaga thailam might be due to its potency to initiate Re- epithelialization.
Conclusion: Thus it is evidenced that Gandhaga thailam has a potential wound healing activity.

Keywords: Siddha; cytotoxicity; wound healing; Gandhagam; mineral based; Scratch; L929.

1. INTRODUCTION

"The disruption in integrity of the skin tissue is generally referred to as a wound" [1]. "Wound healing is a physiological response to a tissue injury. The cascade of initial vasoconstriction of blood vessels and platelet aggregation is designed to stop bleeding" [2]. "This is followed by an influx of a variety of inflammatory cells, starting with the neutrophil. These inflammatory cells, in turn, release a variety of mediators and cytokines to promote angiogenesis, thrombosis, and reepithelialization. The fibroblasts, in turn, lay down extracellular components which will serve as scaffolding. By days 5 through 7, the fibroblasts have started to lay down new collagen and glycosaminoglycan" [3]. "These proteoglycans form the core of the wound and help stabilize the wound. Then, reepithelialization starts to occur with the migration of cells from the wound periphery and adjacent edges. Chronic wounds are those that fail to heal within 4-6 weeks. The primary factors that inhibit wound healing are hypoxia, bacterial colonization, ischemia, reperfusion injury, altered cellular response, and collagen synthesis defects" [4].

"Meanwhile, the long period of management required for chronic wounds, caused by Trauma, pressure, infection, radiation and comorbidities that alter the wound healing process such as diabetes mellitus and peripheral vascular diseases, significantly increase the financial burden of healthcare" [5]. "Damage to the skin affects the quality of life. Chronic wounds continue to be of international and local concern and are an indicator of healthcare quality that generates substantial morbidity and considerable healthcare cost. High costs of treatment can deter patients from seeking care, potentially leading to the development of complex or chronic wounds, In India, public healthcare funding has been reported at 5% of the annual gross domestic product, with a majority (approximately 80%) of healthcare costs met from out-of-pocket payments. A cost effective medicine for wound healing with better prognosis would reduce the economic burden and simultaneously would improve the quality of life of the affected individual" [6].

Siddha system of medicine is nestled with 32 types of Internal medications and 32 types of External medications that can be widely used for various ailments. The reverence of Siddha system could be validated by the copious existing external medications. Vediuppu nitrate), Manosilai (Arsenic disulphide), Navacharam (Ammonium Chloride), and Veeram (Mercuric per chloride) triturated with cow ghee. Eloquence of Gandhaga thailam is because of its preparation which is a special procedure called Gandhaga sudar thailam [7]. Its effect is also facilitated by its mode of administration that, it can be used as both internal medicine and also for topical application.

Sulfur is converted to its oxide or sulphide forms when subjected to purification. This hinders its toxicity and increases its potency [8]. "Sulfur is biologically active element that has been used in dermatology for centuries. It has antibacterial, antifungal, antiviral, and Keratolytic activities besides its anti-tumor activity in the biomedicine field. Sulfur is used for management of different dermatological diseases such as, scabies, acne, and dandruff" [9]. "Potassium nitrate in combination with Silver nitrate has been used for wart removal and ulcer debridement and has been used for postpartum eye infections, mucus membrane infections and burn wounds" [10]. "Various medicines prepared from arsenic disulphide are used for treatment of chronic

fevers and known to possess antimicrobial and antioxidant properties" [11]. "Hydroxy propyltrimethyl Ammonium Chloride Chitosan exhibited effective antibacterial activity towards both Gram-positive (*S. aureus*) and Gram-negative (*P. aeruginosa*) bacteria" [12].

Although all the ingredients of the mentioned oil possess several important medicinal values, the scientific validation for each of its properties is lacking. This is the first study to validate the antioxidant, antibacterial and wound healing potency of Gandhaga thailam. There is a myth persisting ever that heavy metals cause toxicity rather than accepting their therapeutic efficacy. Siddha system is unique in using various heavy metals in all possible ways of treating deadlier diseases in a simple and effective way. All the metals are not used as such in their raw form. They are purified properly in order to alter their metallic form and increase their bioavailability. These mineral and metal based medicines possess zero adverse effects when taken properly with prescribed adjuvant and stipulated time duration. Gandhaga thailam is being used in siddha system to be effective on both skin diseases and the restoration of cutaneous homeostasis. In addition Gandhaga thailam is medicated oil prepared in a special method called sudar thailam rather than normal oils. All the above mentioned minerals are mixed with cow's ghee and the mixture is burnt in direct fire and collected as droplets which is specifically called as sudar thailam.

Primary objective of this study is to validate the wound healing potency of the siddha formulation Gandhaga thailam through scratch wound healing assay.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents Used

I. Process of preparation of Gandhaga thailam [7]

Gandhagam (Sulphur) Vediuppu, (Potassium Nitrate) and Manosilai (Arsenic di Sulphide) 35 gram each are Powdered. Navacharam (Ammonium chloride), Veeram (Mercuric Per Chloride) 2.5 gram each are then added. Then they are triturated with Cow ghee (140 gram) to get the Thailam by doing the Sudar Thailam Procedure. Dosage is about 488 mg, twice a day for 10 days.

II. Reagents

Dulbecco's modified Eagles medium (DMEM Gibco, Invitrogen)), Fetal Bovine Serum - Gibco, US origin,0.25% Trypsin - Invitrogen, USA 25200-056, MTT - Sigma Aldrich M5655,L-glutamine, sodium bicarbonate (Merck, Germany)

2.2 *In vitro* Cytotoxic Assay

L929 (Mouse fibroblast) cell line was cultured in a 25 cm 2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate, and antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphoteracin B (2.5µg/ml). Cultured cell lines were kept at 37ºC in a humidified 5% CO 2 incubator (NBS Eppendorf, Germany). The viability of cells was evaluated by direct observation of cells by Inverted phase contrast microscope and followed by the MTT assay method.

2.3 Cells Seeding in 96 Well Plates

Two days old confluent monolayer of cells was trypsinized and the cells were suspended in 10% growth medium, 100µl cell suspension (5x10 3 cells/well) was seeded in 96 well tissue culture plate and incubated at 37ºC in a humidified 5% CO 2 incubator.

2.4 Preparation of Compound Stock

"1mg of sample was weighed and dissolved in 1mL 0.1% DMSO using a cyclomixer. The sample solution was filtered through a 0.22 µm Millipore syringe filter to ensure sterility. After 24 hours the growth medium was removed, and freshly prepared each compound inDMEM was five times serially diluted by two-fold dilution (100µg, 50µg, 25µg, 12.5µg, 6.25µg in 500µl of DMEM) and each concentration of 100µl was added in triplicates to the respective wells and incubated at 37ºC in a humidified 5% CO 2 incubator. Non-treated control cells were also maintained" [13].

2.5 Cytotoxicity Assay by Direct Microscopic Observation

"The entire plate was observed after 24 hours of treatment in an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera) and microscopic observations were recorded as images. Any

detectable changes in the morphology of the cells, such as rounding or shrinking of cells, granulation, and vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity" [14].

2.6 Cytotoxicity Assay by MTT Method

Fifteen mg of MTT (Sigma, M-5655) was reconstituted in 3 ml PBS until completely dissolved and sterilized by filter sterilization. After 24 hours of the incubation period, the sample content in wells was removed and 30µl of reconstituted MTT solution was added to all test and cell control wells, the plate was gently shaken well, then incubated at 37ºC in a humidified 5% CO 2 incubator for 4 hours. After the incubation period, the supernatant was removed, 100µl of MTT Solution was added and the wells were mixed gently by pipetting up and down to solubilize the formazan crystals. The absorbance values were measured by using a microplate reader at a wavelength of 540 nm [15].

The percentage of growth inhibition was calculated using the formula:

$$\% \text{ of viability} = \frac{\text{Mean OD Samples x } 100}{\text{Mean OD of the control group}}$$

2.7 *In vitro* Scratch Wound Healing Assay

L929 cell lines were initially procured from the National Centre for Cell Sciences (NCCS), Pune, India, and maintained Dulbecos modified Eagles medium. The cell line was cultured in a 25 cm^2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate, and antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphoteracin B (2.5µg/ml). Cultured cell lines were kept at 37ºC in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany).

Exponentially growing cells were trypsinized and seeded at a density of 200,000 cells per well into a 12-well plate for 24 h incubation (~90% confluence) [16]. The scratch wounds were made by a sterile 1 mL pipette tip through a pre-marked line. After removal of the resulting debris from five lineal scratches, the cell monolayer was subsequently rinsed three times with PBS followed by incubation with a sample volume of 25 µg/mL from the stock solution for 0 hours, 24 hours, 48 hours, and 72 hours.

The wound areas were displayed by taking images just above the interchanges between scratched wound areas and pre-marked lines and the effect of the sample on wound closure was determined microscopically (4X magnification, Olympus CKX41) after incubation. The effect of the sample on wound closure was measured in terms of area using MRI-ImageJ analysis software.

2.8 Statistical Analysis

All experiments were done in triplicates and all data were tabulated and represented as Mean+/-SE. One-way ANOVA and Dunnets test were performed to analyze the significance.

3. RESULTS AND DISCUSSION

3.1 Cytotoxicity Assay

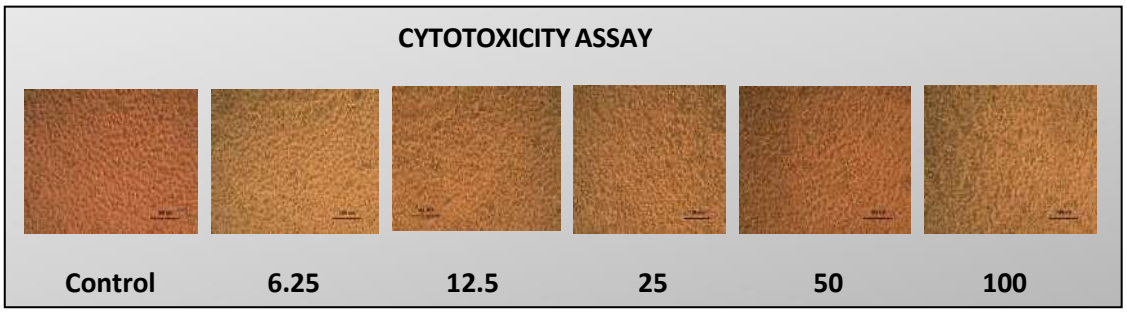
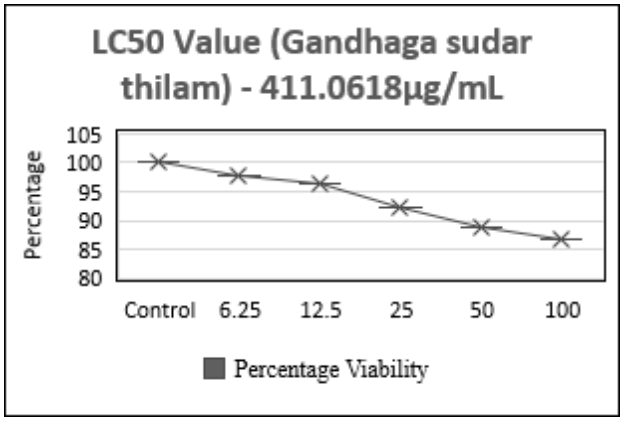


Fig. 1. Light microscopy images of the L929cells in the presence of control and dose-dependent effect of Gandhaga thailam on L929 cells at 6.25,12.5,25,50,100 µg/mL, respectively after 24 h (Magnification 10x, Scale bar: 100 µm). Absorbance @ 540 nm

3.2 Percentage of Cell Viability

a.



b.

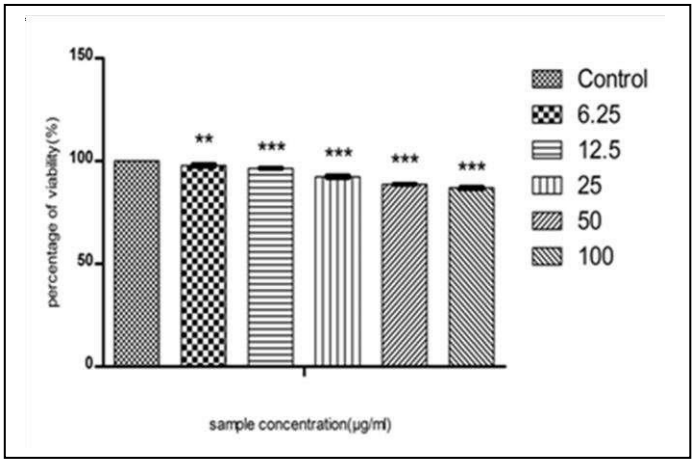
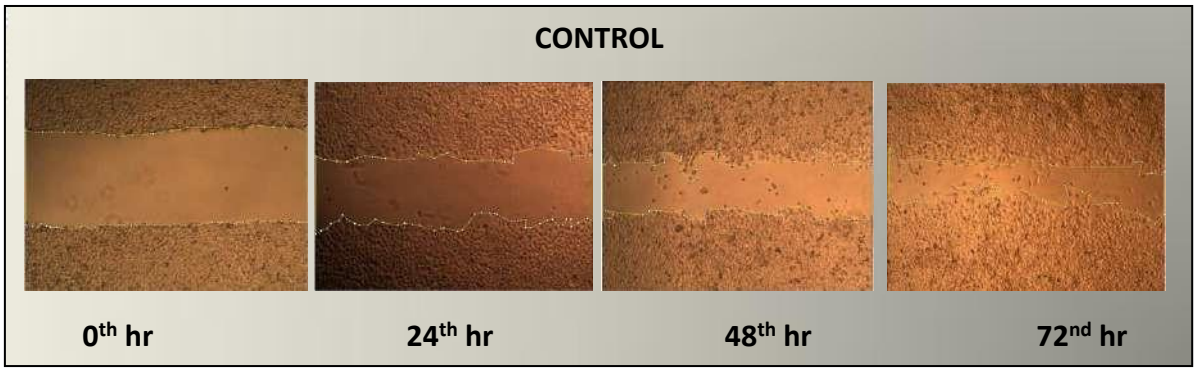


Fig. 2a and b. Graphical representation depicting the dose dependent cytotoxicity effect of Gandhaga thailam on L929 cells by MTT assay. Along Y axis Percentage viability, Along the X-axis varied concentrations of Gandhaga thilam

*The data is the mean from three independent experiments and expressed as the mean ± standard error. ***p < 0.001 compared to control groups, **p < 0.01 compared to control groups. LC 50 Value of Gandhaga thailam is Calculated using ED50 PLUS V1.0 Software*

3.3 Wound Scoring

a)



b)

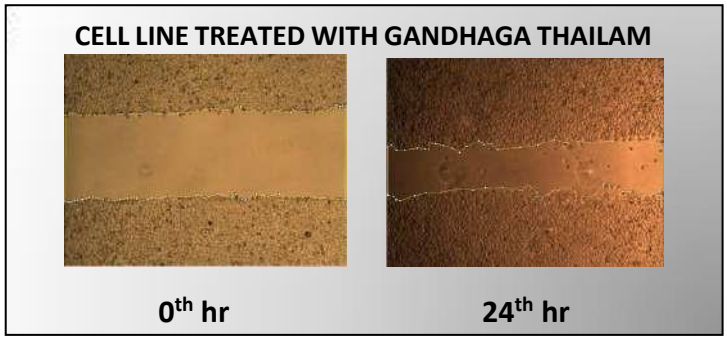


Fig. 3. Light microscopy images of the scratch of the mouse dermal fibroblast L929 cell and migration after the 0th, 24th,48th and 72nd hrs. Magnification 10x Scale bar: 100 µm. a) control b) Gandhaga thailam

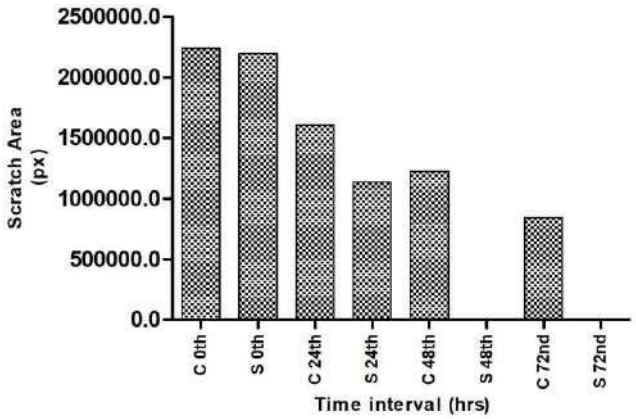


Fig. 4. Graphical representation to compare the Effect of the Gandhaga thailam and untreated (Control) on mouse dermal fibroblast L929 cell lines and the area of scratch due to migratory and proliferative activities of fibroblasts in the scratch assay after 0th, 24th,48th and 72nd hrs of incubation

Table 1. Comparison of the Effect of the Gandhaga thailam and Control - mouse dermal fibroblast L929 cell lines on the area of scratch at constant time intervals (1 px=1/96inch)

Time interval (Hrs)	Wound area (px)
C 0th	2236644
S 0th	2195863
C 24th	1606110
S 24th	1133940
C 48th	1224801
S 48th	0
C 72nd	842379
S 72nd	0

Measurement of two-dimensional cell migration can be done using the wound healing (or scratch) assessment. On a confluent cell monolayer, an artificial gap is created, and movement is monitored with a microscope. Additionally, the scratch test is a practical and affordable primary method to rule out a drug's ability to cure wounds [17]. The second stage of wound healing, which is marked by keratinocyte or fibroblast migration and proliferation, is addressed by the scratch assay [18,19,20].

Keratinocytes are essential for the process of reepethelialization. In addition to covering the wound, the keratinocytes migrate towards the scratch and proliferate to create an epithelial covering. The tissue integrity is preserved through the proliferation of epithelim, which aids in reconstructing the skin's full thickness. Certain growth factors, such as TGF-α (transforming growth factor-α), KGF (keratinocyte growth factor), and EGF (epithelial growth factor),

maintain proliferation. Integrins also aid in the process of Re- epithelialization [21].

As far as we are aware, this is the first report on the Gandhaga thailam and its wound healing activity. In addition to the wound healing experiment, we investigated at the possible cytotoxic effects of Gandhaga thailam on the L929 (Mouse fibroblast) cell line. Furthermore, evaluation of toxicity is an essential aspect of pharmaceutical preparation and its quality assurance [22].

The percentage of cell viability at 6.25 µg/mL, 12.5 µg/mL, 25 µg/mL, 50 µg/mL, and 100 µg/mL of Gandhaga thailam is 97.8%, 96.4%, 92.3%, 88.6%, and 86.9%, respectively, as indicated in the Fig. 1. The Siddha protocol states that plant extracts can be used to convert hazardous mercury into a safe therapeutic medicine, as proven by a number of earlier research [23,24]. Purification of Mercury is used for converting the toxic element mercury into a drug with antioxidant activities [25]. It is well established that conventional medicinal formulations are effective at eliminating the toxicity from metallic substances to create safe herbo mineral medications [26,27].

As Gandhaga thailam is recommended in the siddha system for wound healing, our first hypothesis was that it would promote collagen metabolism and encourage granulation at the wound site. A Traditional Siddha Formulation for Skin Disorders has already been discussed with HPLC study by Adithya et al. [28].

From the results, it was clear that Gandhaga thailam caused a rise in cell number and wound closure in the scratch assay, which showed up earlier in the 48th hour than in the control [Figs. 3 & 4]. This work demonstrates the value of the scratch assay in providing preliminary information on the capacity of Gandhaga thailam to heal damaged dermis, even if it cannot completely replace in vivo studies as a definitive proof of efficacy in wound healing.

The potential wound healing effect of Gandhaga thailam may be due to its antioxidant and anti-inflammatory property that is to be ruled out in future studies.

4. CONCLUSION

In conclusion, Gandhaga thailam possess a good wound healing effect in mouse dermal fibroblast L929 cell lines. The wound closure and formation of fibroblast is much faster due to its rich anti-inflammatory and antioxidant property. Our results can be a starting point for further studies aiming at the interpretation of the molecular processes and signalling pathways underlying proliferation and migration of the fibroblasts induced by Gandhaga thailam.

CONSENT AND ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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