

# INVITROWOUNDHEALINGEFFECTOF A SIDDHA FORMULATION–GANDHAGATHAILAM

## ABSTRACT:

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. Gandhagathailam (Medicated oil), a siddha formulation prepared from (sulphur) Gandhagam, (Potassium nitrate) Vediuppu, (Arsenic disulphide) Manosilai, (Ammonium Chloride) Navacharam, and (Mercuric per chloride) Veeram triturated with cow's ghee is used in a variety of skin diseases. Scientific validation of the wound healing efficacy of Gandhagathailam remains unveiled. The main objective of this research is to validate the wound healing efficacy of Gandhagathailam through invitro studies in L929 (Mouse fibroblast) cell line. The results of the study show that Gandhagathailam is efficient in wound healing and the wound is closed by about 48<sup>th</sup> hour after the scratch. Thus it is evidenced that Gandhagathailam has a potential wound healing activity.

## INTRODUCTION:

The disruption in integrity of the malfunction of the skin tissue is generally referred to as a wound [Eming SA et al, 2014]. Wound healing is a physiological response to tissue injury. The cascade of initial vasoconstriction of blood vessels and platelet aggregation is designed to stop bleeding [Periyah MH et al., 2017]. 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 [Pawel Olczyk et al., 2014]. 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 [Prakash Monika et al., 2021].

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 [Frykberg RG 2015]. 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 [Prakash TV 2016].

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. One such medicine is our test drug Gnadhagathailam (medicated oil) that possess its own effective medicinal values validated by its ingredients namely (sulphur) Gandhagam, (Potassium

nitrate )Vediuppu ,(Arsenic disulphide ) Manosilai, (Ammonium Chloride) Navacharam , and(Mercuricperchloride)Veeramtrituatedwithcow'sghee.EloquenceofGandhagathailamisbecauseofitspreparationwhichisaspecialprocedurecalledGandhagasudarthailam[Agasthiar Paripooranam400 ].Its effectisalsofacilitatedbyits mode ofadministration that ,itcan beusedasbothinternal medicineand alsofortopicalapplication.

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 [Hashem N Metal., 2021]. 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 [Hermans, M.H., 2006]. Various medicines prepared from arsenic disulphide are used for treatment of chronic fevers and known to possess antimicrobial and antioxidant properties [Ramkumar, G., 2014]. Hydroxy propyltrimethyl Ammonium Chloride Chitosan exhibited effective antibacterial activity towards both Gram-positive (*S. aureus*) and Gram-negative (*P. aeruginosa*) bacteria [Yang S, et al., 2017].

Although all the ingredients of the mentioned oil possess several important medicinal values, the scientific validation for each of its properties are lacking. This is the first study to validate the antioxidant , antibacterial and wound healing potency of Gandhagathailam.

There is a myth persisting ever that the 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 deadly 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. Gandhagathailam is being used in Siddha system to be effective on both skin diseases and the restoration of cutaneous homeostasis. In addition Gandhagathailam is medicated oil prepared in a special method called sudarthailam 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 sudarthailam.

Primary objective of this study is to validate the wound healing potency of the Siddha formulation Gandhagathailam through scratch wound healing assay.

## **MATERIALS AND METHODS:**

### **CHEMICALS AND REAGENTS USED:**

#### **I. Process of preparation of Gandhagathailam [Agasthiar Paripooranam 400]:**

Gandhagam (Sulphur) VEDIUPPU, (Potassium Nitrate) and Manosilai (Arsenic disulphide) 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's butter (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 Eagle's 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)

### **INVITRO CYTOTOXIC ASSAY**

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

#### **Cells seeding in 96 well plates:**

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

#### **Preparation of compound stock:**

1 mg of sample was weighed and dissolved in 1 mL 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 in

DMEM 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 triplicate to the respective wells and incubated at 37°C in a humidified 5% CO<sub>2</sub> incubator. Non-treated control cells were also maintained (Jerard C et al., 2020).

### **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 (Mosmann T. 1983).

### **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 37°C in a humidified 5% CO<sub>2</sub> 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 540nm (Laura B. Talarico et al., 2004).

The percentage of growth inhibition was calculated using the formula:

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

### **In Vitro Scratch Wound Healing Assay:**

L929 cell lines were initially procured from the National Centre for Cell Sciences (NCCS), Pune, India, and maintained in Dulbecco's modified Eagle's medium. The cell line was cultured in a 25 cm<sup>2</sup> tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate, and antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphotericin B (2.5µg/ml). Cultured cell lines were kept at 37°C in a humidified 5% CO<sub>2</sub> 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) (Ramachandran et al., 2017). 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 linear scratches, the cell monolayer was subsequently rinsed three times with PBS followed by incubation with a sample volume of 25µg/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.

## STATISTICAL ANALYSIS:

All experiments were done in triplicates and all data were tabulated and represented as Mean ± SE. One-way ANOVA and Dunnett's test were performed to analyze the significance. **RESULTS:**

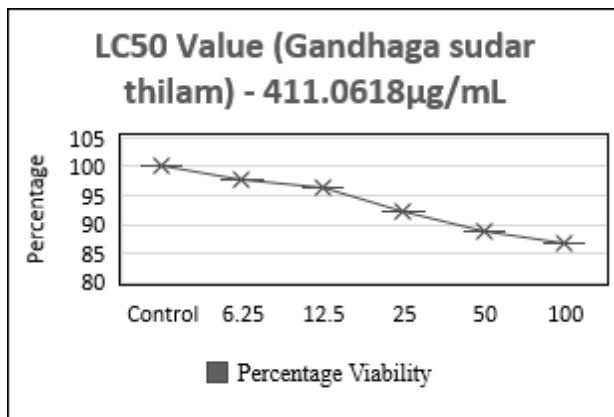
### Cytotoxicity Assay:



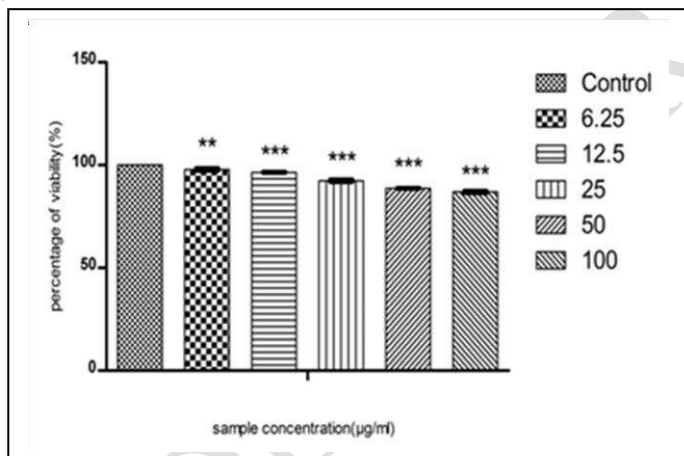
Fig. 1 Light microscopy images of the L929 cells in the presence of control and dose-dependent effect of Gandhagathailam on L929 cells at 6.25, 12.5, 25, 50, 100  $\mu\text{g}/\text{mL}$ , respectively after 24 h (Magnification 10x, Scale bar : 100  $\mu\text{m}$ ). Absorbance @ 540 nm

## Percentage of cell viability

a.



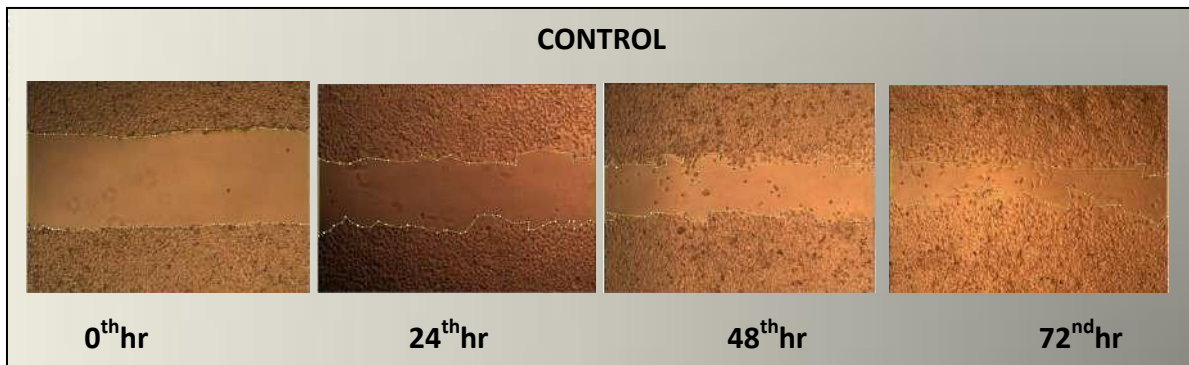
b.



**Figure 2 : a and b** Graphical representation depicting the dose dependent cytotoxicity effect of Gandhagathailam on L929 cells by MTT assay. Along Y axis Percentage viability, Along the X-axis varied concentrations of Gandhagathailam. The data is the mean from three independent experiments and expressed as the mean  $\pm$  standard error. \*\*\* $p < 0.001$  compared to control groups, \*\* $p < 0.01$  compared to control groups. LC50 Value of Gandhagathailam is Calculated using ED50PLUS V1.0 Software.

## Woundscoring

a)



b)

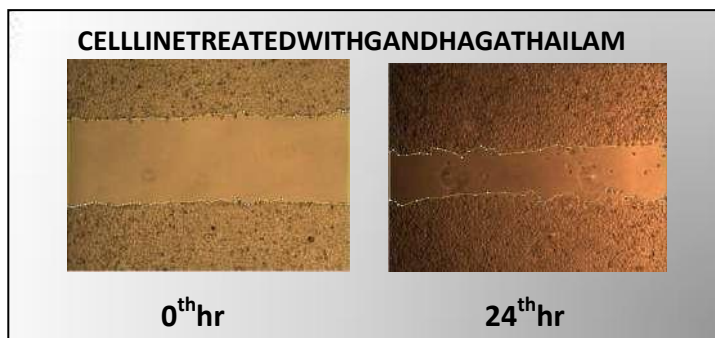


Fig. 3 Light microscopy images of the scratch of the mouse dermal fibroblast L929 cell and migration after the 0<sup>th</sup>, 24<sup>th</sup>, 48<sup>th</sup> and 72<sup>nd</sup> hrs. Magnification 10x Scale bar: 100  $\mu$ m. a) control b) Gandhagathailam

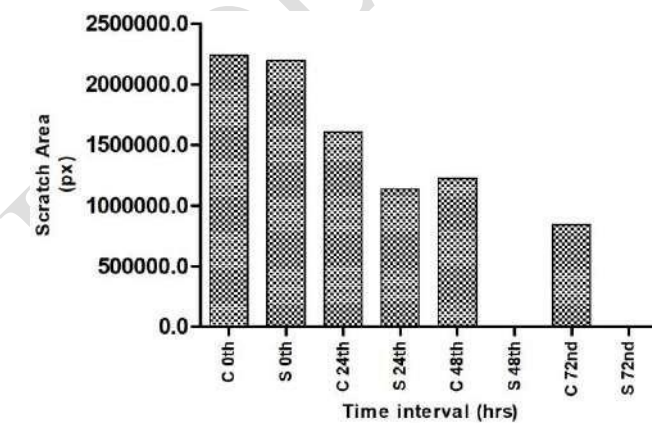


Fig.4 Graphical representation to compare the Effect of the Gandhagathailam and untreated (Control) on mouse dermal fibroblast L929 cell lines and the area of scratch due

tomigratoryandproliferativeactivitiesoffibroblastsinthescratchassayafter0<sup>th</sup>,24<sup>th</sup>,48<sup>th</sup>and72<sup>nd</sup>hrsof incubation

**Table1:**ComparisonoftheEffectoftheGandhagathailamandControl-mousedermalfibroblastL929celllineson theareaofscratch atconstant timeintervals(1px=1/96inch)

<b>TIMEINTERVAL (Hrs)</b>	<b>WOUNDAREA (px)</b>
C 0th	2236644
S 0th	2195863
C 24th	1606110
S 24th	1133940
C 48th	1224801
S 48th	0
C 72nd	842379
S 72nd	0

## Discussion:

Measurement of two-dimensional cell migration can be done using the wound healing (orscratch)assessment.Ona confluent cell monolayer,anartificial gapis created,andmovement is monitored with a microscope. Additionally, the scratch test is a practical andaffordable primary method to rule out a drug's ability to cure wounds. [M. Fronza et al 2009].The second stage of wound healing, which is marked by keratinocyte or fibroblast migrationand proliferation, is addressed by the scratch assay. (Martin, 1997; Schafer and Werner, 2007;Gurtneretal., 2008)

Keratinocytes are essential for the process of reepithelialization. In addition to coveringthewound,the keratinocytes migrate towardsthescratchandproliferatetocreateanepithelial covering. The tissue integrity is preserved through the proliferation of epithelium,which aids in reconstructing the skin's full thickness. Certain growth factors, such as TGF- $\alpha$ (transforminggrowthfactor- $\alpha$ ),KGF(keratinocytegrowthfactor),andEGF(epithelial

growth factor), maintain proliferation. Integrins also aid in the process of reepithelialization. [Cellular and molecular, Clark RA]

As far as we are aware, this is the first report on the Gandhagathailam and its wound healing activity. In addition to the wound healing experiment, we investigated at the possible cytotoxic effects of Gandhagathailam on the

L929 (Mouse fibroblast) cell line. Furthermore, evaluation of toxicity is an essential aspect of pharmaceutical preparation and its quality assurance. [Kaptaner Igci And Aytaç 2020]

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 Gandhagathailam is 97.8%, 96.4 %, 92.3 %, 88.6%, and 86.9%, respectively, as indicated in the figure 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. (Kiefer et al., 2019). Gokarn et al. also validated the efficiency of the age-old procedure of converting the toxic element mercury into a drug with antioxidant activities (Arunachalam, 2015). It is well established that conventional medicinal formulations are effective at eliminating the toxicity from metallic substances to create safe herbomineral medications. (Al-Ansari et al., 2021, Sujatha Pushpakanthi Hewageegana et al., 2021).

As Gandhagathailam 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.

From the results, it was clear that Gandhagathailam 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 [Fig. 3 & 4]. This work demonstrates the value of the scratch assay in providing preliminary information on the capacity of Gandhagathailam 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 Gandhagathailam may be due to its antioxidant and anti-inflammatory property that is to be ruled out in future studies.

## Conclusion

In conclusion, Gandhagathailam possesses 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 Gandhagathailam.

## References:

1. Eming SA, Martin P, Tomic-Canic M. Wound repair and regeneration: mechanisms, signaling, and translation. *Sci Transl Med.* 2014;6(265):265sr6. doi:10.1126/scitranslmed.3009337
2. Periyah MH, Halim AS, Mat Saad AZ. Mechanism Action of Platelets and Crucial Blood Coagulation Pathways in Hemostasis. *Int J Hematol Oncol Stem Cell Res.* 2017;11(4):319-327.
3. **Pawel Olczyk**, Łukasz Mencner, and Katarzyna Komosinska-Vassev The Role of the Extracellular Matrix Components in Cutaneous Wound Healing *BioMed Research International* Volume 2014 doi.org/10.1155/2014/747584
4. Prakash monika et al., Challenges in healing wound: role of complementary and alternative medicine *Front. Nutr.*, 20 January 2022 Sec. Clinical Nutrition Volume 8 -2021 | <https://doi.org/10.3389/fnut.2021.791899>
5. Frykberg RG, Banks J. Challenges in the Treatment of Chronic Wounds. *Adv Wound Care (New Rochelle)*. 2015;4(9):560-582. doi:10.1089/wound.2015.0635
6. Prakash TV, Chaudhary DA, Purushothaman S, K V S, Arvind K V. Epidermal Grafting for Chronic Complex Wounds in India: A Case Series. *Cureus.* 2016;8(3):e516. Published 2016 Mar 1. doi:10.7759/cureus.516
7. Agasthiar Paripooranam 400; Author: R.M.K. Thirumalaisamy Pillai; Published in: 1994; Page no. 155, 156.
8. Hashem NM, Hosny AEMS, Abdelrahman AA, Zakeer S. Antimicrobial activities encountered by sulfuranoparticles combating *Staphylococcus* species harboring SCCmecA recovered from acne vulgaris. *AIMS Microbiol.* 2021 Nov 30;7(4):481-498. doi:10.3934/microbiol.2021029.
9. Hermans, M.H.. (2006). Silver-containing dressings and the need for evidence: Before choosing a specific dressing, take a look at the literature. *American Journal of Nursing*. 106. 91-95.
10. Ramkumar, G. (2014). Acute and 28 Days Repeated Oral Toxicity Studies of ASiddha Drug Kalamega Narayana Chendooram in Kodiveli Verpattai Chooranam on Wistar Albino Rats. 5. 46-51.

11. Yang S, Han X, Jia Y, Zhang H, Tang T. Hydroxypropyltrimethyl Ammonium Chloride Chitosan Functionalized-PLGA Electrospun Fibrous Membranes as Antibacterial Wound Dressing: In Vitro and In Vivo Evaluation. *Polymers (Basel)*. 2017 Dec 11;9(12):697. doi: 10.3390/polym9120697.
12. Jerard C, Michael BP, Chenicheri S, Vijayakumar N, Ramachandran R. Rosmarinic Acid-Rich Fraction from *Mentha arvensis* Synchronizes Bcl/Bax Expression and Induces G<sub>0</sub>/G<sub>1</sub> Arrest in Hepatocarcinoma Cells. *Proc Natl Acad Sci, India, Sect B Biol Sci*. 2020 Sep;90(3):515–22.
13. Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*. 1983 Dec;65(1–2):55–63
14. Talarico LB, Zibetti RG, Faria PC, Scolaro LA, Duarte ME, Nosedá MD, Pujol CA, Damonte EB. Anti-herpes simplex virus activity of sulfated galactans from the red seaweeds *Gymnogongrus griffithsiae* and *Cryptonemiacrenulata*. *Int J Biol Macromol*. 2004 Apr;34(1-2):63-71. doi:10.1016/j.ijbiomac.2004.03.002.
15. Ramachandran R, Saraswathi M. Postconditioning with metformin attenuates apoptotic events in cardiomyoblasts associated with ischemic reperfusion injury. *Cardiovascular Therapeutics*. 2017 Dec;35(6):e12279.
16. Cellular and molecular facets of keratinocyte reepithelization during wound healing, *Experimental Cell Research*, Volume 304, Issue 1, 2005, Pages 274-286, ISSN 0014-4827, <https://doi.org/10.1016/j.yexcr.2004.10.033>
17. Clark RA, editor. *The molecular and cellular biology of wound repair*. Springer Science & Business Media; 2013 Nov 11.
18. Kaptaner İgci and Aytaç An Investigation on the In Vitro Wound Healing Activity and Phytochemical Composition of *Hypericum pseudolaevae* N. Robson Growing in Turkey. *Turk J Pharm Sci* 2020;17(6):610-619 DOI:10.4274/tjps.galenos.2019.80037
19. Martin, P., 1997. Wound healing – aiming for perfect skin regeneration. *Science* 276,75–81.
20. Schafer, M., Werner, S., 2007. Transcriptional control of wound repair. *Annual Review of Cell and Developmental Biology* 23, 69–92.
21. Gurtner, G.C., Werner, S., Barrandon, Y., Longaker, M.T., 2008. Wound repair and regeneration. *Nature* 453,314–321.

22. M. Fronza et al., Determination of the wound healing effect of Calendula extracts using the scratch assay with 3T3 fibroblasts, *Journal of Ethnopharmacology* 126(2009)463–467
23. Kiefer A.M., Seney C.S., Boyd E.A., Smith C., Shivdat D.S., Matthews E., Hull M.W., Bridges C.C., Castleberry A., Mukherjee A. Chemical analysis of Hg<sup>0</sup>-containing Hindu religious objects. *PLoS ONE*. 2019;14(12):e0226855.

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