INVITROWOUNDHEALINGEFFECTOF A SIDDHAFORMULATION–GANDHAGATHAILAM

ABSTRACT:

Wound is defined as disruption in the integrity of skin. Wound healing is a physiologicalprocess primarily initiated by vasoconstriction and platelet aggregation. Wound closure iscompleted by release of inflammatory markers and aggregation of fibroblasts which causesreepitheliazation .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 withcow's ghee is used in a variety of skin diseases.Scientific validation of the wound healingefficacy of Gandhagathailam remains unveiled.The main objective of this research is tovalidate the wound healing efficacy of Gandhagathailam through invitro studies inL929(Mousefibroblast)cellline.TheresultsofthestudyshowsthatGandhagathailamisefficientin wound healing and the wound is closed by about 48th hour after the scratch. Thus it isevidencedthatGandhagathailam hasapotentialwoundhealingactivity.

Comment [DN1]: Better to write siddha name first followed by chemical name in brackets (eg: Gandhagam (Sulphur)

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INTRODUCTION:

The disruption in integrity of the malfunction of the skin tissue is generally referred toas a wound [Eming SA et al, 2014].Wound healing is a physiological response to atissueinjury. The cascade of initial vasoconstriction of blood vessels and platelet aggregation isdesignedtostopbleeding[PeriayahMHetal.,2017].Thisisfollowedbyaninfluxofavariety of inflammatory cells, starting with the neutrophil. These inflammatory cells, in turn,releaseavarietyofmediatorsandcytokinestopromoteangiogenesis,thrombosis,andreepitheli alization. The fibroblasts, in turn, lay down extracellular components which willserve as scaffolding. By days 5 through 7, the fibroblasts have started to lay down newcollagen and glycosaminoglycan [Pawel Olczyk et al., 2014]. These proteoglycans form thecore of the wound and help stabilize the wound. Then, reepithelialization starts to occur withthe migration of cells from the wound periphery and adjacent edges. Chronic wounds arethose that fail to heal within 4-6 weeks. The primary factors that inhibit wound healing arehypoxia, bacterial colonization, ischemia, reperfusion injury, altered cellular response, andcollagensynthesis defects[Prakashmonika et al .,2021]

Meanwhile, the long period of management required for chronic wounds, caused byTrauma ,pressure , infection ,radiation andcomorbidities that alter the wound healingprocess such as diabetes mellitus and peripheral vascular diseases, significantly increase thefinancial burden of healthcare [Frykberg RG2015]. Damage to the skin affects the quality oflife. Chronic wounds continue to be of international and local concern and are an indicator ofhealthcare quality that generates substantial morbidity and considerable healthcare cost.
Highcostsoftreatmentcandeterpatientsfromseekingcare,potentiallyleadingtothedevelopment of complex or chronic wounds, In India, public healthcare funding has beenreported at 5% of the annual gross domestic product, with a majority (approximately 80%) ofhealthcare costs met from out-of-pocket payments. A cost effective medicine for woundhealing with better prognosis would reduce the economic burden and simultaneously wouldimprovethe qualityof lifeof theaffected individual[PrakashTV2016].

Siddha system of medicine is nestled with 32 types of Internal medications and 32 typesof Externalmedicationsthatcanbewidely used forvariousailments. ThereverenceofSiddha system could be validated by the copious existing external medications. One suchmedicine is our test drug Gnadhagathailam(medicated oil) that possess its own effectivemedicinalvaluesvalidatedbyitsingredientsnamely(sulphur)Gandhagam,(Potassium

Comment [DN2]: Pls recheck

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

Comment [DN3]: Change as indicated in abstract

Sulfur is biologically active element that has been used in dermatology for centuries. Ithas antibacterial, antifungal, antiviral, and Keratolytic activities besides its anti-tumor activityin the biomedicine field. Sulfur is used for management of different dermatological diseasessuchas,scabies,acne,anddandruff[HashemNMetal.,2021].Potassiumnitrateincombination with Silver nitrate has been used for wart removal andulcer debridementandhas been used for postpartum eye infections, mucus membrane infections and burn wounds[Hermans, M.H..2006] .Various medicines prepared from arsenic disulphide are used fortreatment of chronic fevers and known to possess antimocrobial and antioxidant properties[Ramkumar,G.2014].Hydroxy propyltrimethyl Ammonium Chloride Chitosanexhibitedeffective antibacterial activity towards both Gram-positive (S. aureus) and Gram-negative (P.aeruginosa)bacteria[YangS,etal.,2017]

Although all the ingredients of the mentioned oil possess several important medicinalvalues, the scientific validation for each of its properties are lacking. This is the first study tovalidate the antioxidant ,antibacterial and wound healing potency of Gandhagathailam. Thereisamythpersistingeverthatheavymetalscausetoxicityratherthanacceptingtheirtherapeutic efficacy. Siddha system is unique in using various heavy metals in all possiblewaysoftreatingdeadlierdiseasesinasimpleandeffectiveway.Allthemetalsarenotusedassuch in their raw form .They are purified properly in order to alter their metallic form andincrease their bioavailabilty. These mineral and metal based medicines possess zero adverseeffects when taken properly with prescribed adjuvant and stipulated time duration. Gandhagathailamisbeingusedinsiddhasystemtobeeffectiveonbothskindiseasesandtherestorationofcutaneoushomeostasis.InadditionGandhagathailamismedicatedoilprepared in a special method called sudarthailam rather than normal oils. All the abovementioned minerals are mixed with cow's ghee and the mixture is burnt in direct fire andcollectedasdroplets which is specificallycalled assudarthailam.

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PrimaryobjectiveofthisstudyistovalidatethewoundhealingpotencyofthesiddhaformulationGandhagathailamthrough scratch woundhealingassay.

MATERIALSANDMETHODS:

CHEMICALSANDREAGENTSUSED:

I. **ProcessofpreparationofGandhagathailam**[AgasthiarParipooranam400]**:**

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'sbutter(140gram)togettheThailambydoingtheSudarThailamProcedure.Dosageisabout488 mg,Twiceadayfor10 days.

II. Reagents:

Dulbecco'smodifiedEaglesmedium(DMEMGibco,Invitrogen)),FetalBovineSerum-Gibco,USorigin,0.25%Trypsin-Invitrogen,USA 25200-056,MTT-SigmaAldrichM5655,L-glutamine,sodium bicarbonate(Merck,Germany)

INVITROCYTOTOXICASSAY

L929 (Mouse fibroblast) cell line was cultured in a 25 cm 2 tissue culture flask withDMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate, and antibioticsolution 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 (NBSEppendorf, Germany).The viability of cells was evaluated by direct observation of cells byInvertedphasecontrastmicroscopeandfollowedbytheMTTassaymethod.

Cellsseedingin96wellplates:

Twodaysoldconfluentmonolayerofcellswastrypsinizedandthecellsweresuspendedin 10% growth medium, 100µl cell suspension (5x10 3 cells/well) was seeded in 96 welltissuecultureplateandincubatedat37ºC inahumidified 5%CO2 incubator.

Preparationofcompoundstock:

1mgofsamplewasweighedanddissolvedin1mL0.1%DMSOusingacyclomixer.The

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sample solution was filtered through a 0.22 µm Millipore syringe filter to ensure sterility.After24hoursthegrowthmediumwasremoved, andfreshlypreparedeachcompound in

DMEM was five times serially diluted by two-fold dilution (100µg, 50µg, 25µg, 12.5µg,6.25µgin500µlof DMEM)andeach concentrationof100µlwasaddedintriplicatesto the respectivewellsandincubatedat37ºCinahumidified5% CO_2incubator. Non-treatedcontrolcellswerealso maintained (JerardC et al.,2020).

CytotoxicityAssaybyDirectMicroscopicObservation:

The entire plate was observed after 24 hours of treatment in an inverted phase contrasttissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera) andmicroscopic observations were recorded as images.Anydetectablechangesinthe morphology of the cells, such as rounding or shrinking of cells, granulation, andvacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity(MosmannT.1983).

CytotoxicityAssaybyMTTMethod:

FifteenmgofMTT(Sigma, M-5655)wasreconstituted in3 mlPBSuntil completely dissolved and sterilized by filter sterilization. After 24 hours of the incubation period, thesample content in wells was removed and 30µl of reconstituted MTT solution was added toall test and cell control wells, the plate was gently shaken well, then incubated at 37ºC in ahumidified 5% CO_2 incubator for 4 hours. After the incubation period, the supernatant wasremoved, 100µl of MTT Solution was added and the wells were mixed gently by pipetting upanddowntosolubilizetheformazancrystals.Theabsorbancevalues were measuredbyusingamicroplatereaderata wavelengthof540nm(LauraB.Talaricoetal.,2004).

Thepercentageof growthinhibitionwascalculatedusingtheformula:

$$\%ofviability = \frac{MeanODSamplesx100}{MeanODofthecontrolgroup}$$

InVitroScratchWoundHealingAssay:

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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 ina 25 cm^2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodiumbicarbonate, and antibiotic solution containing: Penicillin (100U/ml), Streptomycin(100µg/ml), and Amphoteracin B (2.5µg/ml). Cultured cell lines were kept at 37ºC in ahumidified5%CO_2incubator (NBSEppendorf,Germany).

Exponentially growing cells were trypsinized and seeded at a density of 200,000 cells perwell 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. Afterremoval of the resulting debris from five lineal scratches, the cell monolayer wassubsequently rinsed three times with PBS followed by incubation with a sample volume of 25µg/mLfrom the stocksolution for 0 hours,24 hours, 48hours,and72 hours.

The wound areas were displayed by taking images just above the interchanges betweenscratched wound areas and pre-marked lines and the effect of the sample on wound closurewasdeterminedmicroscopically(4Xmagnification,OlympusCKX41)afterincubation.The effect of the sample on wound closure was measured in terms of area using MRI-ImageJanalysissoftware.

STATISTICALANALYSIS:

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

RESULTS:

CytotoxicityAssay:

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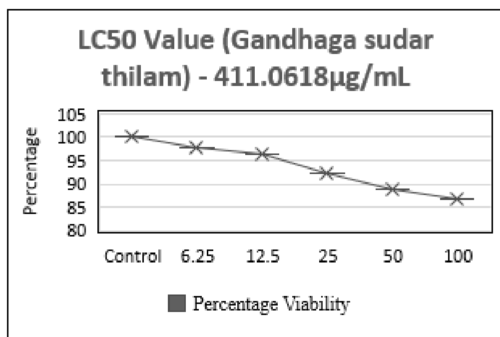
Fig. 1Light microscopy images of theL929cells in the presence of control and dose-dependenteffectofGandhagathailamonL929cellsat6.25,12.5,25,50,100µg/mL ,respectivelyafter24 h (Magnification10x, Scalebar :100µm).Absorbance@540 nm

Percentageofcellviability

a.



b.

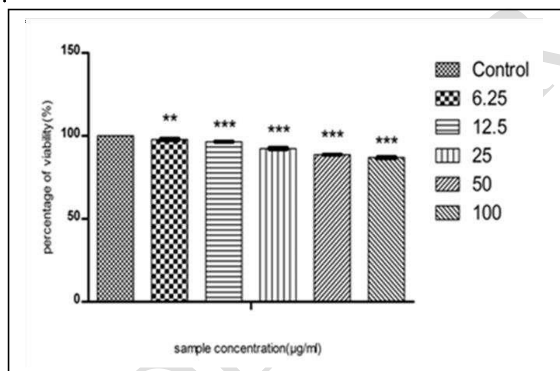


Figure2 : a and b Graphical representation depicting the dose dependent cytotoxicity effectof Gandhagathailam on L929 cells by MTT assay. Along Y axis Percentage viability, Alongthe X-axis varied concentrations of Gandhagathilam .The data is the mean from threeindependent experiments and expressed as the mean ± standard error. ***$p < 0.001$ comparedtocontrolgroups,**$p<0.01$comparedtocontrolgroups.LC50ValueofGandhagathailam isCalculated usingED50PLUSV1.0Software.

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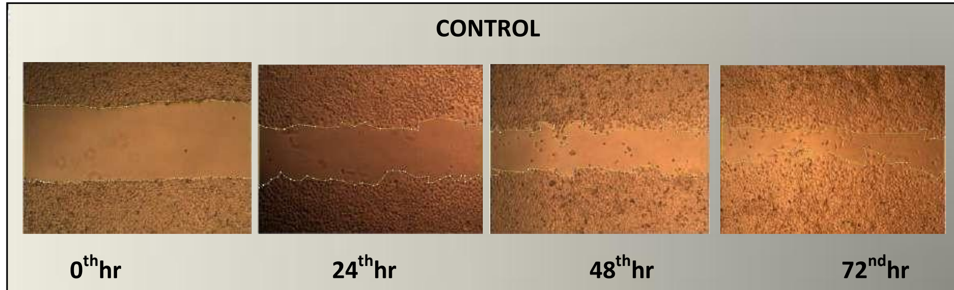
Woundscoring

a)



b)

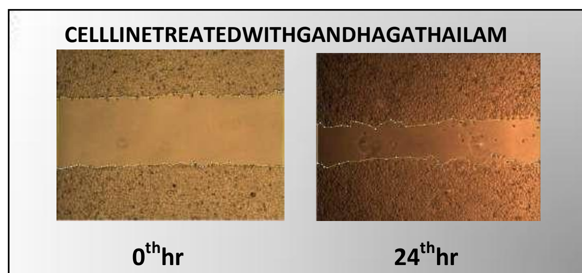


Fig. 3 Light microscopy images of the scratch of the mouse dermal fibroblast L929 celland migration after the 0th, 24th,48th and 72nd hrs. Magnification 10x Scale bar: 100 μm. a)controlb)Gandhagathailam

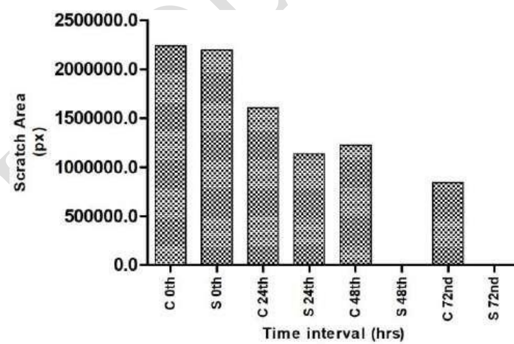


Fig.4GraphicalrepresentationtocomparetheEffectoftheGandhagathailamanduntreated(Control) onmousedermalfibroblastL929celllinesandtheareaofscratchdue

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tomigratoryandproliferativeactivitiesoffibroblastsinthescratchassayafter0th,24th,48thand72ndhrsof incubation

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

TIMEINTERVAL (Hrs)	WOUNDAREA (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

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Discussion:

Measurement of two-dimensional cell migration can be done using the wound healing (orscratch)assessment.Onaconfluentcellmonolayer,anartificialgapiscreated,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 reepethelialization. In addition to coveringthewound,thekeratinocytesmigratetowardsthescratchandproliferatetocreateanepithelial 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(transforminggrowthfactor-α),KGF(keratinocytegrowthfactor),andEGF(epithelial

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

As far as we are aware, this is the first report on the Gandhagathailam and itswoundhealing activity. In addition to the wound healing experiment, we investigated at the possiblecytotoxiceffectsofGandhagathailamonthe L929(Mousefibroblast)cellline.Furthermore,evaluationoftoxicityisanessentialaspectofpharmaceuticalpreparationanditsqualityassurance. [KaptanerIgciAndAytaç2020]

Thepercentageofcellviabilityat6.25µg/mL,12.5µg/mL,25µg/mL,50µg/mL,and100 µg/mL of Gandhagathailam is 97.8%, 96.4 %, 92.3 %, 88.6%, and 86.9%, respectively, asindicated in the figure1. TheSiddha protocol states that plant extracts can be used to converthazardousmercuryintoasafetherapeuticmedicine,asprovenbyanumberofearlierresearch.(Kiefer et al., 2019).Gokarn et al. also validated the efficiency of the age-oldprocedure of converting the toxic element mercury into a drug with antioxidant activities(Arunachalam, 2015). It is well established that conventional medicinal formulations areeffective at eliminating the toxicity from metallic substances to create safe herbomineralmedications.(Al-Ansarietal.,2021,SujathaPushpakanthiHewageeganaetal.,2021).

As Gandhagathailam is recommended in the siddha system for wound healing, our firsthypothesis was that it would promote collagen metabolism and encourage granulation at thewoundsite.

From the results, it was clear that Gandhagathailam caused a rise in cell number and woundclosure 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 preliminaryinformation on the capacity of Gandhagathailam to heal damaged dermis, even if it cannotcompletelyreplacein vivostudies asadefinitiveproofof efficacyin woundhealing.

ThepotentialwoundhealingeffectofGandhagathailammaybeduetoitsantioxidantandanti-inflammatorypropertythat is to be ruled out in futurestudies.

Conclusion

In conclusion, Gandhagathailampossess a good wound healing effect inmouse dermalfibroblast L929 cell lines. The wound closure and formation of fibroblast is much faster duetoitsrichanti-inflammatoryandantioxidantproperty.Ourresultscanbeastartingpointfor

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furtherstudiesaimingattheinterpretationofthemolecularprocessesandsignallingpathways underlying proliferation and migration of the fibroblasts induced byGandhagathailam.

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