www.siddhapapers.org
ISSN 0974-2522

Siddha Papers 2016 (11) (1)
Research Article

In-Vitro wound healing activity of Herbal topical formulation on H9C2 Heart cells

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ABSTRACT

The Need of Traditional Medical treatments are gradually increasing day by day because of raising awareness about our deep rooted traditions, adverse effects of current treatment methods etc. On the other side, healing wounds will be considered as an art of Medicine. With new technologies, we are still facing many down drifts in healing the wounds especially in patients with diabetes. So the authors', tried to develop a new Poly-herbal formulation and introduced the current formulation for the treatment of diabetic ulcers especially foot ulcers. Here we carried out the *In-vitro* Wound healing Scratch assay on H9C2 Heart cells, the results show significant healing activity and area of wound closure when compared to the existing control. Thus the study suggests, that the current formulation has been successful in treating diabetic wounds through In-vitro studies and further studies are needed on a large scale involving clinical trials to explore this more.

Keywords

Wound healing, Scratch wound assay, Diabetic foot ulcer, Herbal wound treatment, Siddha Medicine, In-vitro assay.

INTRODUCTION

Wound healing is a complex and dynamic process of replacing the missing cellular structures and tissue layers. Healing is the interaction of complex cascade of cellular events that generates resurfacing, reconstitution and restoration of strength of injured tissue. In wound healing, Inflammation, Proliferation and maturation are the major three classical steps. The poly coded herbal formulation is mainly composed of rich wound healing activity drugs, formulated by the expert committee of Walters Siddha Research Centre (WSRC), Tirunelveli. The same poly coded formula was prepared as per SOP (Standard operative Procedure) and also carried out Institutional clinical trials with respect to ethical committee approval from WSRC.

MATERIALS AND METHODS

Exponentially growing H9C2 cells were trypsinized and seeded at a density of 200,000 cells per well into 12-well plates for 24 h incubation (∼90% confluence). The scratch wounds were made by a sterile 10L pipette tip through a pre-marked line. After removal of the resulting debris from five lineal scratches, H9C2 monolayer was subsequently rinsed three times with PBS followed by incubated with PBS containing indicated concentrations of HA for 30 min. After rinsed with PBS, cell were further incubated in 400 m H2O2 for 24 h and rinsed three times with PBS. The wound areas were displayed by taking images just above the interchanges between scratched wound areas and pre-marked lines.

Sample preparation and 2D-DIGE-based proteomic analysis:

The detail experimental procedures have been described in our previous publications (Lai et al., 2010). Briefly, H9C2 cells in normal growth medium at ∼80% confluence were used for proteomic analysis. For total cellular protein analysis, cells with various treatments were washed in chilled 0.5 × PBS and scraped in 2-DE lysis buffer containing 4% (w/v) CHAPS, 7 M urea, 2 M thiourea, 10 mMTris–HCl, pH 8.3, 1 mM EDTA. Lysates were homogenized by passage through a 25-gauge needle 10 times, insoluble material was removed by centrifugation at 13,000 rpm for 30 min at 4 ◦C, and protein concentrations were

determined by using Coomassie Protein Assay Reagent (BioRad). Before performing 2D-DIGE, protein samples were labelled with N-hydroxysuccinimidyl ester-derivatives of the cyanine dyes Cy2, Cy3 and Cy5 following the protocol described previously (Wu et al., 2012). Briefly, 150g of protein sample was minimally labelled with 375pmol of either Cy3 or Cy5 for comparison on the same 2-DE. To facilitate image matching and cross-gel statistical comparison, a pool of all samples was also prepared and labeled with Cy2 at a molar ratio of 2.5 pmol Cy2 per g of protein as an internal standard for all gels. Thus, the triplicate samples and the internal standard could be run and quantify on multiple 2-DE. The labeling reactions were performed in the dark on ice for 30 min and then quenched with a 20-fold molar ratio excess of free l-lysine to dye for 10 min. The differentially Cy3- and Cy5-labeled samples were then mixed with the Cy2-labeled internal standard and reduced with dithiothreitol for 10 min. IPG buffer, pH 3–10 nonlinear 2%, v/v (GE Healthcare) was added and the final volume was adjusted to 450 L with 2D-lysis buffer for rehydration. The rehydration process was performed with immobilized non-linear pH gradient (IPG) strips (pH 3–10, 24 cm) which were later rehydrated by CyDyelabeled samples in the dark at room temperature overnight (at least 12 h). Isoelectric focusing was then performed using a Multiphor II apparatus (GE Healthcare) for a total of 62.5 kV-h at 20 ◦C. Strips were equilibrated in 6 M urea, 30% (v/v) glycerol, 1% SDS (w/v), 100 mMTris–HCl (pH 8.8), 65 mMdithiothreitol for 15 min and then in the same buffer containing 240 mMiodoacetamide for another 15 min. The equilibrated IPG strips were transferred onto 26 cm ×20 cm 12.5% poly-acrylamide gels casted between low fluorescent glass plates. The strips were overlaid with 0.5% (w/v) low melting point agarose in a running buffer containing bromophenol blue. The gels were run in an Ettan Twelve gel tank (GE Healthcare) at 4.5 W per gel at 10 ◦C until the dye front had completely run off the bottom of the gels. Afterward, the fluorescence 2-DE was scanned directly between the low fluorescent glass plates using an Ettan DIGE Imager (GE Healthcare). This imager is a charge-coupled device-based instrument that enables scanning at different wavelengths for Cy2-, Cy3-, and Cy5-labeled samples. Gel analysis was performed using DeCy-der 2-D Differential Analysis Software v7.0 (GE Healthcare) to co-detect, normalize and quantify the protein features in the images. Features detected from non-protein sources (e.g. dust particles and dirty backgrounds) were filtered out. Spots displaying aaverage-fold increase or decrease in abundance with a p-value < 0.05 were selected for protein identification.

Protein staining, in-gel digestion and MALDI-TOF MS analysis

Colloidal coomassie blue G-250 staining was used to visualize CyDye-labeled protein features in 2-DE followed by excised interested post-stained gel pieces for MALDI-TOF MS identification. The detailed procedures for protein staining, in-gel digestion, MALDI-TOF MS analysis and the algorithm used for data processing were described in our previous publication (Lai et al., 2010). The spectrometer was also calibrated with a peptide calibration standard (Bruker Daltonics) and internal calibration was performed using trypsin autolysis peaks at m/z 842.51 and m/z 2211.10. Peaks in the mass range of m/z 800–3000 were used to generate a peptide mass fingerprint that was searched against the Swiss-Prot/TrEMBL database (v57.12) with 513,877 entries using Mascot software v2.2.06 (Matrix Science, London, UK). The following parameters were used for the search: Rodent; tryptic digest with a maximum of 1 missed cleavage; carbamido-methylation of cysteine, partial protein N-terminal acetylation, partial methionine oxidation and partial modification of glutamine to pyroglutamate and a mass tolerance of 50 ppm. Identification was accepted based on significant MASCOT Mowse scores ($p < 0.05$), spectrum annotation and observed versus expected molecular weight and pI on 2-DE.

Western Blot Analysis

Cells were cultured at a density of cells/cm2 per plate. After treatment with H2O2 for 1 h, cells were washed once with cold PBS and then lysed with RIPA buffer (50 mMTris, 150 mMNaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, sodium orthovanadate, sodium fluoride, EDTA, and leupeptin) and 1 mM phenylmethylsulfonyl fluoride (PMSF) for 15 min on ice. Soluble proteins were collected by centrifugation at 5500 ×g for 5 min. The protein concentration in each sample was determined using a BCA protein assay kit (with BSA as a standard). For immunoblotting, 80 μg of protein were loaded onto a 15% SDS-polyacrylamide gel electrophoresis (PAGE) and subsequently transferred onto a polyvinylidene difluoride (PVDF) membrane. Adequate transfer of protein was confirmed by Coomassie Blue staining of the gel and Ponceau Red staining of the membranes. Equal protein loading was confirmed by probing for β-actin. After blocking with 7% skim milk, the membranes were probed with respective primary antibodies (1: 1000 dilution). The membranes were subsequently probed with horseradish peroxidase-conjugated anti-rabbit antibody (1 : 1500). Proteins were detected using enhanced chemiluminescence ECL Western blotting detection reagent, and bands were visualized by exposure to

photographic film. Densito-metric analysis of protein band was performed by using BandScan 4.0 software.2.8.

Phospho-specific Protein Microarray Analysis

Phospho-specific protein microarray was obtained from Full Moon Biosystems Inc. Protein microarray analysis was carried out using the protocol provided. The procedure used to process the array was as follows: 50 μg of cell lysates in 60 μL of reaction solution were labeled with 3 μL biotin in 10 mg/mL N, N-dimethylformamide. 50 μg of biotin-labeled proteins were diluted in 6 mL of coupling solution and labeled as "Protein Coupling Mix" before being applied to the array for conjugation. To prepare the antibody microarray, it was blocked with blocking solution for 45 min at room temperature, rinsed with Milli-Q grade water. Then the array was incubated with Protein Coupling Mix on an orbital shaker rotating at 35 rpm for 2 h at room temperature. Afterwards, the array slide was washed twice with 30 mL of wash solution for 10 min each, rinsed extensively with Milli-Q water, and then incubated with a Cy3-steptavidin (0.5 mg/mL) for 45 min in the dark at room temperature. This was followed by rinsing steps with Milli-Q water. After drying by centrifugation, the slide was scanned on a GenePix 4000B scanner (Axon Instruments, USA) and the images were analyzed with GenePix Pro 6.0. Fluorescence intensity of each array spot was quantified, and the mean value was calculated. For each treatment group, a phosphorylation signal ratio induction or reduction was calculated based on the following equation: where phosphor A or phosphor B and unphosphoA or unphosphoB were signals of the phosphorylated and non-phosphorylated proteins from the experimental samples, respectively.

Simulated Ischemia (SI):

SimulatedIschemia (SI)will be induced byincubatingcardiomyocytes (H9c2 cell line) with specifiedmodified Krebs-Henseleit buffer (137 mMNaCl, 3.8 mMKCl, 0.49 mM$MgCl_2$, 0.9mM $CaCl_2$, and 4.0 mM HEPES) with 20mM 2-deoxyglucose, 20 mM sodiumlactate and 1 mM sodiumdithionite atpH 6.5. Control buffer composed ofKrebs-Henseleit buffer (137 mMNaCl, 3.8mM KCl, 0.49 mM $MgCl_2$, 0.9 mM $CaCl_2$ and 4.0 mM HEPES) supplemented with20 mM D-glucose, 1 mM sodiumpyruvate. After simulate ischemia will haveachieved, the ischemic buffer or controlbufferwill be removed and the cells will besubjected to reperfusion by the addition of 2ml complete medium before furtherincubating at 37°C, 5% CO_2 for 24 hours.

Measurement of cell viability Assay:

The measurement of H9c2 cardio-myoblastviability will be performed by the reductionof MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) bymitochondrial reductases. At the end ofreperfusion period, the medium will becollected for lactate dehydrogenase (LDH)activity. Cells will be incubated with 0.01g/ml MTT for 2 hours at 37°C. Then, 1ml of 0.04 M HCl in isopropanol will be added to each well. The converted dyewill be collected and the optical density will be determined spectro-photometrically at λ570 nm with background subtraction at λ 650 nm. Cell viability will becalculated as a percentage of control.

Measurements of cellular injury:

The cellular injury of H9c2 cardiomyoblastcell line will be measured based on theextracellular release of lactatedehydrogenase (LDH) and CK, according to theloss of plasma membrane integrity.

Measurement of Creatine Kinase (CK) and Lactate Dehydrogenase (LDH) Activity:

Coronary heart perfusate will be placed on ice after weighinguntil they will be assayed for creatine kinase (CK) andlactate dehydrogenase (LDH) activity. The extent of CKand LDH leakage during the reperfusion period, as anindication of myocardial injury, will be estimated by thepercentage of CK and LDH accumulative activity at eachminute of the reperfusion time (1–15 minutes). In thepresent study, the CK and LDH content will be measuredby commercially available kits.

For measuring CK activity, 20 μL of the heart perfusatewill be added to 30°C pre-warmed cuvette containing 1 ml CK reagent and mixed by inversion. After3 min incubation, the absorbance will be read at 340 nm,versus water as a reference. Absorbancewill be measuredat 30-second intervals for a period of 120 seconds. Theactivity of CK in U/l is the change of absorbance per minute (ΔA/min).

For LDH measurement, 50 μLof the heart perfusatewill be added to 30°C pre-warmed cuvette containing 1 mlCK reagent and mixed by inversion. After 3 min incubation,the absorbance will be read at 340 nm versus water asa reference. Absorbance will be read at 10-second

intervalsfor a period of 60 seconds. The activity of LDH in U/l isthe change of absorbance per minute (ΔA/min).

Results & Discussion

The test drug shows maximum results in a very little dosage (Fig 1). The *In-vitro* scratch healing assay shows, at 50 µg, 100 µg the area of intensity will be at 35644, 17929 respectively when compared to the control 23323. The area of wound closure (Table. 1) will be raised in 100 µg.

Fig 1. Phase contrast of Wound closure

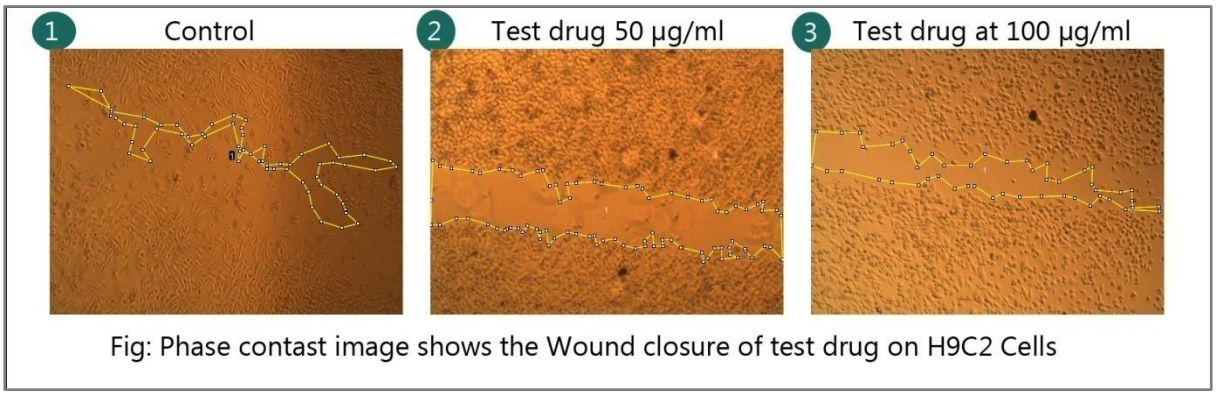


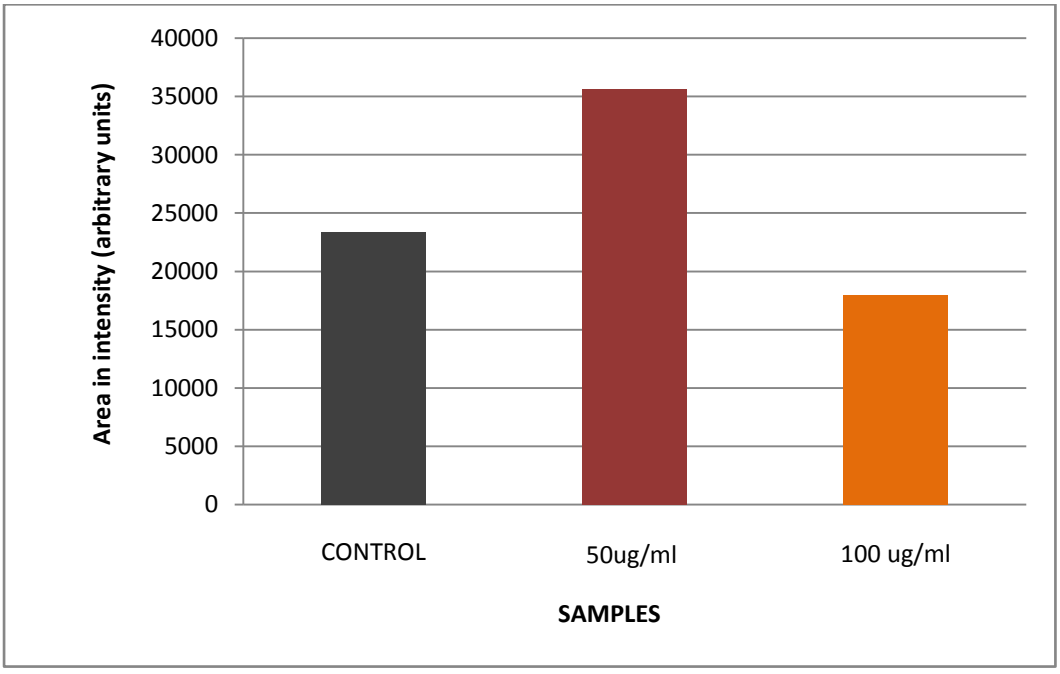
Fig: Phase contast image shows the Wound closure of test drug on H9C2 Cells

Fig 1: Wound closure after 24 hours of extract treatment- (MRI wound healing tool- ImageJ analysis software)

Test	Area of intensity (Arbitrary units)	Area of Wound closure
Control	23323	**121.930**
50 ug	35644	165.922
100ug	17929	**142.295**

Fig. 2 shows the Wound closure after 24 hours of extract treatment and it is visualized under MRI wound healing tool- ImageJ analysis software. Where the plotting made by Along Y axis – Area in intensity (arbitrary units), Along X axis – Samples. 100ug of extracts reduced the area of wound suggesting more activity over 50ug.

Fig. 2 Graphical representation of In-vitro Wound Healing Scratch Assay



Conclusion

The present study demonstrated that the herbal topical formulation was effective in healing the wounds. The in -vitro data indicated that wound healing effects of Test drug might be due to the regulation and coordination of inflammation, angiogenesis and tissue regeneration. This study gave us good scientific evidence that theherbal formula is a promising therapy for diabetic patients with foot ulcers & chronic ulcerations.

Acknowledgement

My sincere acknowledge to our Chief, Walters Siddha Research Centre, for their timely motivation and funds to carry out this work.

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