PROJECT REQUIREMENT:

- To measure the wound healing capacity on HPDLFS, one test compound was received.

- The sample details as follows:

Sl. No.	Sample Name	Sample code	Cell line
1	IRIMEDADI	S1	HPDLFS
2	Cipladine	Cipladine	HPDLFS

Table1: Details of samples received for the study

BACKGROUND OF THE STUDY:

The scratch wound healing assay 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). This assay is simple, inexpensive and experimental conditions can be easily adjusted for different purposes. The assay can also be used for a high-through put screen platform if an automated system is used (Yarrow and Perlman, 2004).

MATERIALS:

- Cell lines: HPDLFs–Human Primary Periodontal Ligament Fibroblast cells (Isolated from extracted Human Periodontal ligament)
- Antibiotic-Antimycotic solution (Cat No: A002, Himedia)
- Cell culture medium: DMEM high glucose - (#AL111, Himedia)
- Adjustable multichannel pipettes and a pipettor (Benchtop, USA)
- Fetal Bovine Serum(#RM10432, Himedia)
- D-PBS (#TL1006, Himedia)
- Test compound: IRIMEDADI
- Cipladine Mouth wash (Commercially available)
- 12 well cell culture plate (Biolite - Thermo)
- 50 ml centrifuge tubes (# 546043 TORSON)
- 1.5 ml centrifuge tubes (TORSON)
- 10 ml serological pipettes (TORSON)
- 10 to 1000ul tips (TORSON)
- 70% ethanol

EQUIPMENTS:

1. Centrifuge (Remi: R-8^{o}C).
2. Pipettes: 2-10µl, 10-100µl, and 100-1000µl.
3. Inverted Biological Binocular Microscope (Biolinkz)
4. 37°C incubator with humidified atmosphere of 5% CO_2 (Healforce, China)
5. Biosafety Laminar Hood (Healforce, China)

SOFTWARE:

1. Image J
2. Windows Paint

MAINTENANCE OF CELL LINES:

The HPDLFS (Human Periodontal Ligament Fibroblast cells) were isolated from the ligament of Human Periodontal tissue in sterile conditions by enzymatic digestion method. The cells were maintained in DMEM-high glucose media supplemented with 10 % FBS along with the 1% antibiotic-antimycotic solution in the atmosphere of 5% CO_2, 18-20% O_2 at 37^{0}C temperature in the CO_2 incubator and sub-cultured for every 2 days. Passage number -2 was used for the present study.

STEPS FOLLOWED:

1. Grow cells in DMEM with high glucose media supplemented with 10% FBS until the cells reach 70-80% confluence.
2. Seed cells into 12 well tissue culture plate at a density of 0.25 million cells per well, until they reach ~80-100% confluence as a monolayer for the incubation period of 24hrs.
3. After the incubation time, the monolayer was gently and slowly scratched with a new 200ul pipette tip across the centre of the well. While scratching across the surface of the well, the long-axial of the tip should always be perpendicular to the bottom of the well.
4. The resulting gap distance therefore equals to the outer diameter of the end of tip. The gap distance can be adjusted by using different types of tips. Scratch a straight line in one direction.
5. Scratch another straight line perpendicular to the first line to create a cross in each well.
6. After scratching, gently wash the well twice with medium to remove the detached cells.
7. The cells were treated with desired concentrations of given compound prepared in media and incubated at 37^{o}C with 5% Co_2 in the incubator.

8. Grow cells for additional 48 hours (or the time required if different cells are used).
9. The cell images were captured at different time intervals (ex: 0, 24hr.48hr, etc) for different views of the monolayer.
10. The gap distance can be quantitatively evaluated using software such as ImageJ.
11. To reduce variability in results, it's suggested that multiple views of each well should be documented, and each experimental group should be repeated multiple times.

FORMULA USED FOR THE ANALYSIS:

% Wound Healing Scored= (Initial area-Final area)/Initial area *100

RESULTS:

In this study, given test compound (irimedadi) was evaluated to analyse the wound healing efficacy on HPDLFS. The concentrations of the test compound used to treat the cells are as follows:

Sl.No	Culture condition	Cell lines	Concentration treated to cells
1	Untreated	HPDLFS	No treatment
2	Blank	-	Only Media without cells
3	Cipladine	HPDLFS	100ul/ml
4	irimedadi	HPDLFS	100ul/ml

Table 2: Details of drug treatment to respective cell lines used for the study

OBSERVATIONS & CONCLUSIONS:

Wound Area Covered Overlay-HPDLFS			
Incubation	**Untreated**	**Cipladine**	**Irimedadi**
0 hour	349098.1836	345109.4292	347647.9238
24 hour	315535.0344	177138.8406	189155.2768
48 hour	221060.9862	4972.3186	5386.6782

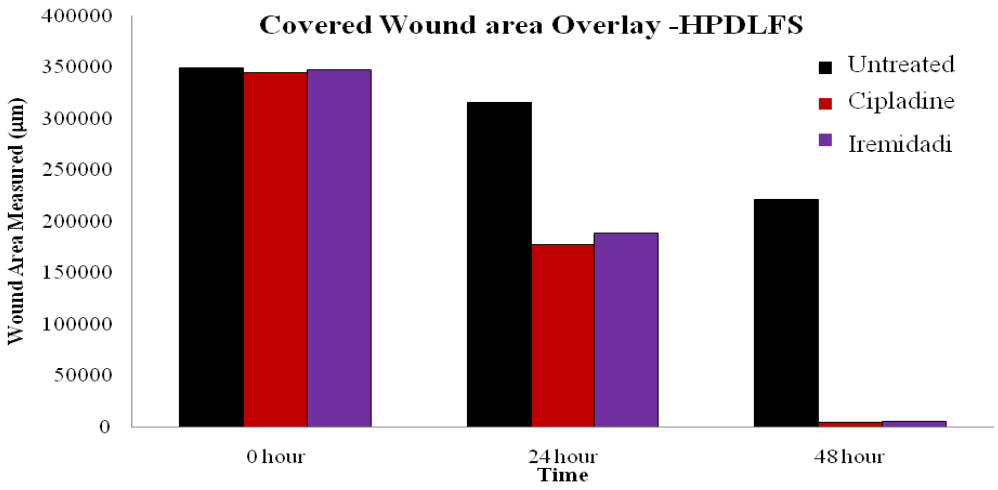
Table-3: Wound healing area values after the treatment period of 0hr, 24hrs and 48hrs.

- The results of Wound healing assay suggest that the given test compound, Irimedadi was effective healing potency in nature on Human Periodontal Ligament Fibroblasts (HPDLFS) which decreased the area of wound on incubation dependent manner respectively.
- The observed wound area with calculations are enclosed in MS excel format.
- The direct microscopic observations of images of test compound (Irimedadi), standard control (Cipladine) after 0 hour, 24hour and 48 hour of incubation were enclosed in the separate folder along with this report.

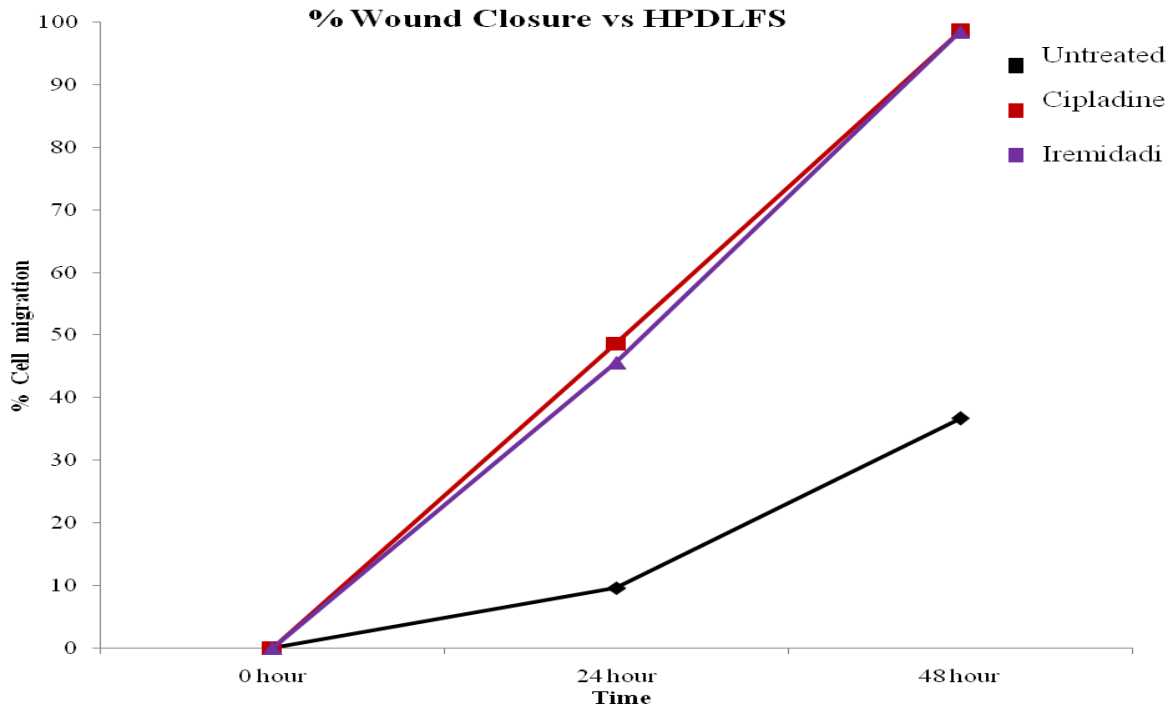
Overlay % Wound Closure scored - HPDLFS			
Incubation	**Untreated**	**Cipladine**	**Irimedadi**
0 hour	0	0	0
12 hour	9.614243435	48.67168915	45.58998807
24 hour	36.67655789	98.55920523	98.45053635

Table-4: % Wound closure values of GAIN-IT treated HPDLFS cells after the treatment period of 24hrs and 48hrs.

OVERLAY GRAPHS:



Graph 1: Measured wound area values of Irimedadi, Cipladine treated cells in comparison to the untreated cells after the different incubation periods of 0 hours, 24hours and 48hours.



Graph 1: % cell migration area of wound closure values of S1 (Irimedadi), Cipladine treated cells in comparison to the untreated cells after the different incubation periods of 0 hour, 24hours and 48hours.

CONCLUSION:

The in-vitro Wound healing assay results suggest us that, the given test compound, Irimedadi was non-toxic as well as cell proliferative in nature on Human Periodontal ligament fibroblasts. After 24hours of incubation, Irimedadi and Cipladine showed wound healing potency of 45.59% and 48.67% respectively where after 48 hours the potency has increased to 98.45% and 98.55% respectively. Irimedadi molecule exhibited effective wound healing properties similar to the commercially available molecule as well as mouth wash, Cipladine and further studies have to be conducted to determine the molecular mechanism behind Wound healing efficacy properties of the test compound at in-vitro level by measuring the level of Collagenase and Elastase by Spectrophotometry method.

Enclosures:

1) **Excel Sheet with Calculations and Raw Data**
2) **Captured Cell Images at different intervals with the measured area**