PROJECT REQUIREMENT:

- To determine the cytotoxicity study on HPDLFs, 1sample was received.
- The sample details as below:

Sl. No.	Sample Name/Code	Concentrations	Cell lines
1	Guduchi Irimedadi	5 (20,40,60,80,100ul/ml)	HPDLFs
2	Chlorhexidine Mouth wash	5 (20,40,60,80,100ul/ml)	HPDLFs
Additional details of compound		Color: Yellowish liquid	

Table1: Details of the Samples received for the study

BACKGROUND OF THE STUDY:

MTT assay is a colorimetric assay used for the determination of cell proliferation and cytotoxicity, based on reduction of the yellow coloured water soluble tetrazolium dye MTT to formazan crystals. Mitochondrial lactate dehydrogenase produced by live cells reduces MTT to insoluble formazan crystals, which upon dissolution into an appropriate solvent exhibits purple colour, the intensity of which is proportional to the number of viable cells and can be measured spectrophotometrically at 570nm. (Alley et al and Mosamann et al).

MATERIALS:

1. Cell lines: HPDLFs–Human Primary Periodontal Ligament Fibroblast cells(Isolated from extracted Human Periodontal ligament)
2. Cell culture medium: DMEM-high glucose medium - (#AL111, Himedia)
3. Adjustable multichannel pipettes and a pipettor (Benchtop, USA)
4. Fetal Bovine Serum (#RM10832, Himedia)
5. Antibiotic-Antimycotic solution (Cat No: A002, Himedia)
6. MTT Reagent (5 mg/ml) (# 4060 Himedia)
7. DMSO (#PHR1309, Sigma)
8. D-PBS (#TL1006, Himedia)

9. Chlorhexidine Mouth wash (Ipka health Products, India)
10. 96-well plate for culturing the cells (From Corning,USA)
11. T25 flask (# 12556009, Biolite - Thermo)
12. 50 ml centrifuge tubes (# 546043 TARSON)
13. 1.5 ml centrifuge tubes (TARSON)
14. 10 ml serological pipettes (TARSON)
15. 10 to 1000 ul tips (TARSON)

EQUIPMENTS:

1. Centrifuge (Remi: R-8C).
2. Pipettes: 2-10µl, 10-100µl, and 100-1000µl.
3. Inverted binocular biological microscope (Biolinkz, India)
4. Biosafety hood (Biobase, China)
5. 37°C incubator with humidified atmosphere of 5% CO_2 (Healforce, China)
6. 96well plate reader (ELX-800, BioTek, CA, USA)

ASSAY CONTROLS:

(i) Medium control (medium without cells)

(ii) Negative control (medium with cells but without the experimental drug/compound)

(iii) Positive control (medium with cells treated with different concentrations of Chlorhexidine).

Note: Extracellular reducing components such as ascorbic acid, cholesterol, alpha-tocopherol, dithiothreitol present in the culture media may reduce the MTT to formazan. To account for this reduction, it is important to use the same medium in control as well as test wells.

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 37^0c temperature in the CO_2 incubator and sub-cultured for every 2days. Passage number -2 was used for the present study.

STEPS FOLLOWED:

1. Seed 200µl cell suspension in a 96-well plate at required cell density (20,000 cells per well), without the test agent. Allow the cells to grow for about 24 hours.
2. Add appropriate concentrations of the test agent.
3. Incubate the plate for 24h at 37°C in a 5% CO_2 atmosphere.
4. After the incubation period, remove spent media and add MTT reagent to a final concentration of 0.5mg/ml of total volume.
5. Wrap the plate with aluminium foil to avoid exposure to light.
6. Return the plates to the incubator and incubate for 3 hours.
7. Remove the MTT reagent and then add 100µl of solubilisation solution (DMSO).
8. Gentle stirring in a gyratory shaker will enhance dissolution. Occasionally, pipetting up and down may be required to completely dissolve the MTT formazan crystals especially in dense cultures.
9. Read the absorbance on a spectrophotometer or an ELISA reader at 570nm wavelength.
10. % Cell viability is calculated using below formula:

% cell viability=[Mean abs of treated cells/Mean abs of Untreated cells] x 100.

RESULTS:

In this study, given test compound was evaluated to analyse the cytotoxicity effect on HPDLFS. The concentrations of the test compound used to treat the cells as follows:

Sl.No	Culture condition	Cell lines	Concentration treated to cells
1	Untreated	HPDLFS	No treatment
2	Blank	-	Only Media without cells
3	Chlorhexidine	HPDLFS	5 (20,40,60,80,100ul/ml)
4	Guduchi Irimedadi	HPDLFS	5 (20,40,60,80,100ul/ml)

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

OBSERVATIONS & CONCLUSIONS:

Culture condition	% cell viability
Untreated	100.00
Guduchi Irimedadi -20µl/ml	99.53
Guduchi Irimedadi -40µl/ml	97.87
Guduchi Irimedadi -60µl/ml	93.49
Guduchi Irimedadi -80µl/ml	88.55
Guduchi Irimedadi -100µl/ml	83.77

Table-3: % cell viability values of HPDLFstreated with given compound, Guduchi Irimedadi after the treatment period of 24hrs.

Culture condition	% cell viability-24hours
Untreated	100.00
Chlorhexidine-20µl/ml	96.26
Chlorhexidine-40µl/ml	91.59
Chlorhexidine-60µl/ml	86.42
Chlorhexidine-80µl/ml	80.14
Chlorhexidine-100µl/ml	66.16

Table-4: % cell viability values of HPDLFstreated with reference std compound, Chlorhexidine mouth wash after the treatment period of 24hrs.

- The results of cytotoxicity study performed by MTT assay suggest that the given test compound, Guduchi Irimedadi was non-toxic in nature on Human Periodontal Ligament fibroblasts with the % cell viability of 83.77% at the highest concentration of 100ul/ml after the incubation period of 24hrs.Commercially available mouth wash, Chlorhexidine caused moderate toxicity on HPDLFs with 66.16% cell viability at the highest concentration of 100ul/ml after the 24hours.
- The absorbance readings with calculations are enclosed in MS excel format.
- The direct microscopic observations of drug treated images of test compound after 24 hours of incubation were enclosed in the separate folder along with this report.

CONCLUSION:

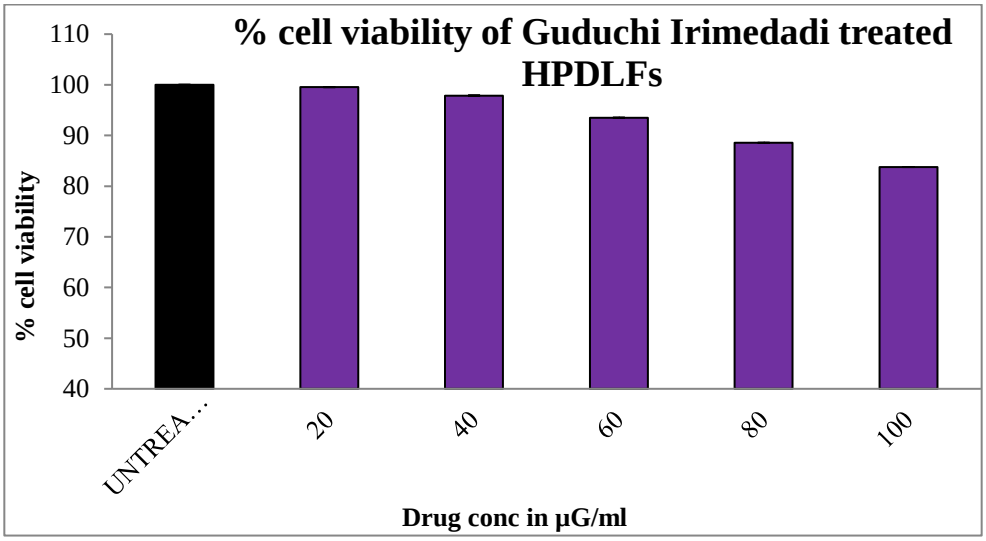
The MTT assay results suggest us that, given test compound, Guduchi Irimedadi was non-cytotoxic on Human Periodontal Ligament Fibroblasts (HPDLFs) with 83.77% cell viability at the highest concentration of given compound at100ul/ml concentration after the treatment period

of 24hrs. Highest concentration of 100ul/ml of compound Guduchi Irimedadi showed significant cell proliferation and can

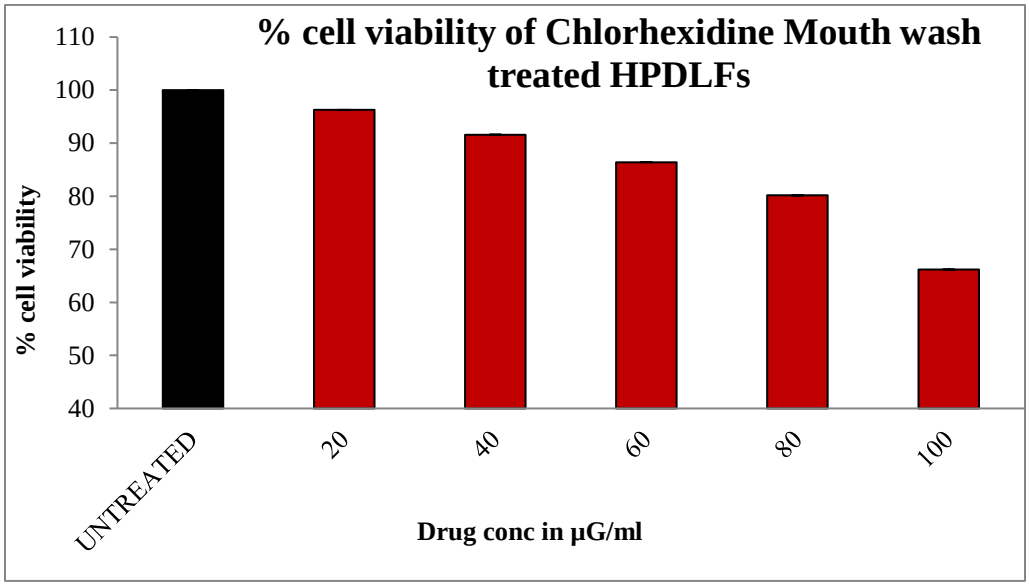
be choose for further assays. Commercially available reference mouth wash, Chlorhexidine caused moderate toxicity on HPDLFs.

However, further studies have to be conducted to determine the molecular mechanism behind cell proliferative properties of the test compound at in-vitro level with studies like Wound healing and growth factor expression studies etc. to find the molecular mechanism of action of test compound in relation to cell proliferation at in-vitro level.

OVERLAY GRAPHS:



Graph 1: % cell viability of HPDLFs treated with Guduchi Irimedadi after the incubation period of 24hrs.



Graph 2: % cell viability of HPDLFs treated with Chlorhexidine mouth wash after the incubation period of 24hrs.

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