7.0 CELL LINE STUDIES

The safety and efficacy of developed formulation was investigated by *in-vitro* cell culture experiments including MTT assay and scratch assay on 3T3 Swiss albino cell line.

7.1 MATERIALS: List of materials used in experiments are shown in table 7.1.

TABLE 7.1: List of materials used in cell line studies

Sr. no.	Materials	Source
1	Dulbecco's modified Eagle's medium,	HiMedia (India)
2	Fetal bovine serum	HiMedia (India)
3	antibiotic antimycotic solutions (10,000 U penicillin and 10 mg Streptomycin per ml in 0.9% normal saline)	HiMedia (India)
4	Trypsin-EDTA solution	HiMedia (India)
5	DMSO (cell culture grade)	HiMedia (India)
6	MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium	HiMedia (India)
7	Elisa plates 96 well	HiMedia (India)
8	Tissue culture plate 6 well	HiMedia (India)
9	25 ml tissue culture flask	HiMedia (India)

Swiss 3T3 albino fibroblasts was purchased from Cell Line Service, NCCS Pune, India and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 0.1% antibiotic solution at 37 ◦C in a humidified atmosphere containing 5% CO2 . Swiss 3T3 albino fibroblast cells are shown in figure 7.1

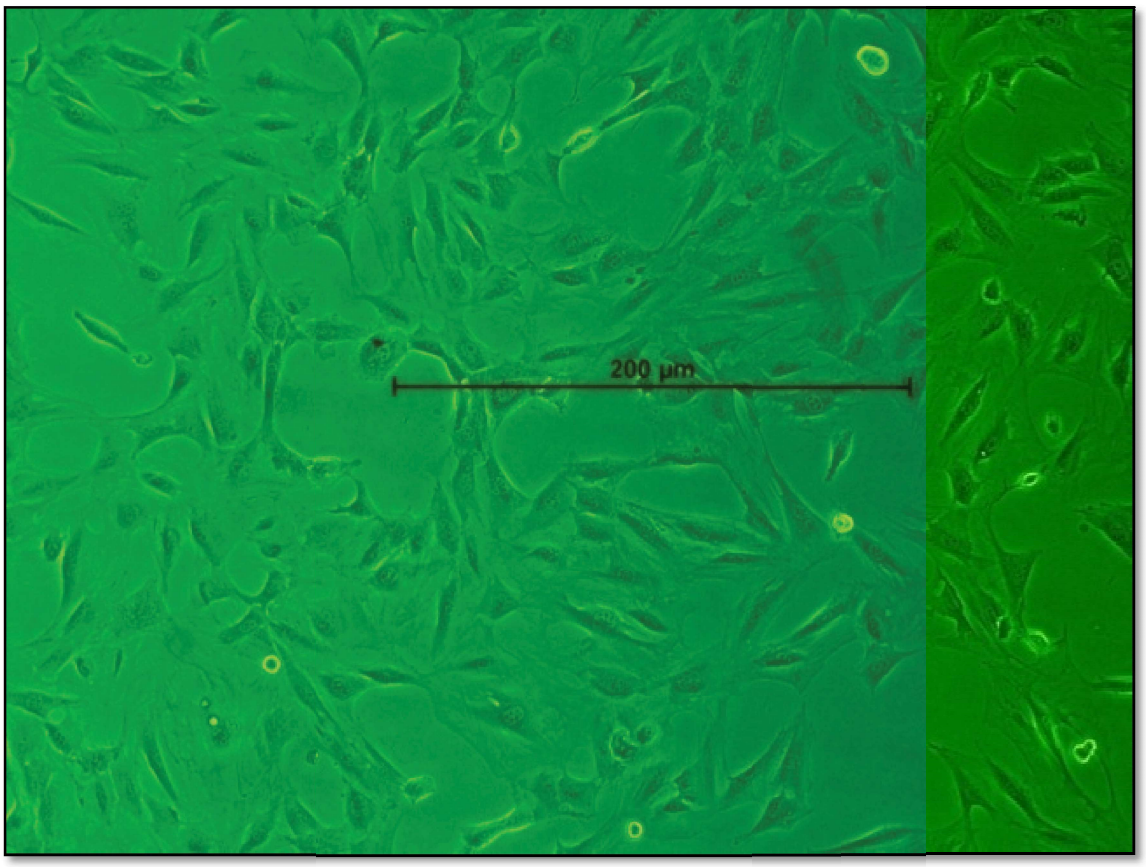

FIGURE 7.1: Swiss 3T3 albino fibroblasts

7.2 *IN-VITRO* CELL CYTOTOXICITY STUDY (MTT ASSAY)

7.2.1 PRINCIPLE:

The MTT assay is colorimetric assay for measuring the activity of enzymes that reduce MTT to formazan, giving a purple color (1). This conversion reaction takes place in mitochondria and so the assay is therefore a measure of mitochondrial activity. Hence, In-*vitro* cytotoxicity of the developed formulation can be evaluated by using MTT assay. Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells. The amount of formazan produced is directly proportional to the viable number of cell in range of cell lines. Lesser the toxicity of the formulation more will be the viability resulting in purple color development after the treatment which can be compared to control. Dimethyl-sulfoxide is added to dissolve the insoluble purple formazan product and make a coloured solution. The absorbance of this coloured solution is then quantified by measuring at 570nm wavelength using ELISA plate reader.

7.2.2 PROCEDURE: The cell cytotoxicity of the prepared formulation and CFE was studied using 3-(4,5-dimethylthizol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (2).

7.2.2.1 Sample preparation: Samples (CFE loaded hydrogel sheet and CFE loaded collagen film) was cut into small pieces and transferred to transfer to sterile falcon tube containing 20.0 ml full culture media. The tubes were incubated for 24 hr at 37° C for extraction. This extract was used for further study.

7.2.2.2 Sub culturing: After 78-80% confluency of cells in culture flask, subculturing was carried out. The complete media from the culture flask was removed aseptically under the laminar air flow. Cells were gently washed thrice with 1.0 ml PBS (pH 7.4) and 1.0 ml of trypsin-EDTA solution was added into that. 2.0 ml of culture media was added after 2 min. 1.5 ml of cell suspension was transferred to new culture flask (25.0 ml) and 4.5 ml culture media was added into that. Culture flask was incubated at 37° C, 5% $CO_2$for 24 hr.

7.2.2.3 MTT assay:

1) About 5 x 10^4 cells were seeded in each well of 96-well plate with 200 µl of complete media and incubated at 37° C, 5% CO_2 for 24 h.
2) Culture medium was removed and cells were washed with PBS.
3) After the cells got adhered at around 24 h, cells were treated with various concentrations of formulations and CFE for 24 h.
4) After the treatment, the media was removed and 10 µL of MTT dye (5 mg/mL) was added to each well containing 100 µL of complete media.
5) The cells were further incubated for 4 h after MTT treatment.
6) Then 100 µL of DMSO was added to each well and incubated for 2 h to allow the solubilization of formazan crystal.
7) Then absorbance was read at 570 nm (ref. Filter of 655 nm) using ELISA plate reader (Bio-Rad, USA).
8) The % cell viability was calculated using Eq (7.1)

$$\% \text{ Cell viability } = \frac{Mean\ absorbance\ (sample)}{Mean\ asborbance\ (control)} * 100 \quad \text{.....Eq (7.1)}$$

7.3 IN-VITRO WOUND HEALING (SCRATCH ASSAY)

The spreading and migration capabilities of Swiss 3T3 fibroblasts were assessed using a scratch wound assay which measures the expansion of a cell population on surfaces(3). The cells were seeded into 6-well tissue culture dishes 24 h at 37° C with 5% CO_2 at a concentration of 3×10^5 cells/ml and cultured in medium containing 10% FBS to nearly confluent cell monolayers. Then, a linear wound was generated in the monolayer with a sterile 200 µl plastic pipette tip. Any cellular debris was removed by washing the cover slips with phosphate buffer saline (PBS). DMEM medium (control group), CFE, formulations extract were added to well and incubated for 24 h and 48 h at 37° C with 5% CO2. Three representative images from each well of the scratched areas under each condition were photographed using inverted microscope (Nikon Eclipse TS100, Japan) to estimate the relative migration of cells.

7.4 RESULTS AND DISCUSSION

7.4.1 *IN-VITRO* CELL CYTOTOXIC STUDY: To specify a non-toxic and appropriate dose, the cytotoxic effects of different concentrations of CFE and formulations on swiss 3T3 albino fibroblasts cells were tested by the MTT assay. Results of in-vitro cell cytotoxicity study are expressed in table 7.2 & figure 7.2 (for CFE loaded hydrogel sheet) and in table 7.3, figure 7.3 (for CFE loaded collagen film). No cytotoxicity activity was observed with both developed formulations and CFE because % cell viability was above 95% with all samples. At the end of incubation with extract of formulation and CFE, there was increase in cell viabilities compared to the positive control groups. It might be due to stimulation of cell proliferation by CFE (4).

TABLE 7.2: % Cell viability of hydrogel sheet with CFE and CFE in MTT assay

Sample Name	**Average absorbance at 570 nm (ref. filter of 655 nm) (±SD, n=8)**	**% Cell viability (±SD, n=8)**
Only media (control)	0.2640 ± 0.0307	100 ± 11.6287
CFE (250 ppm)	0.2842 ± 0.03629	117.6136 ± 8.0675
CFE (500 ppm)	0.3205 ± 0.02292	121.1015 ± 11.3229
Hydrogel sheet loaded with CFE (~ 250 ppm CFE)	0.2720 ± 0.01356	103.0303 ± 4.9852
Hydrogel sheet loaded with CFE (~ 500 ppm CFE)	0.2790 ± 0.03208	105.6818 ± 11.4982

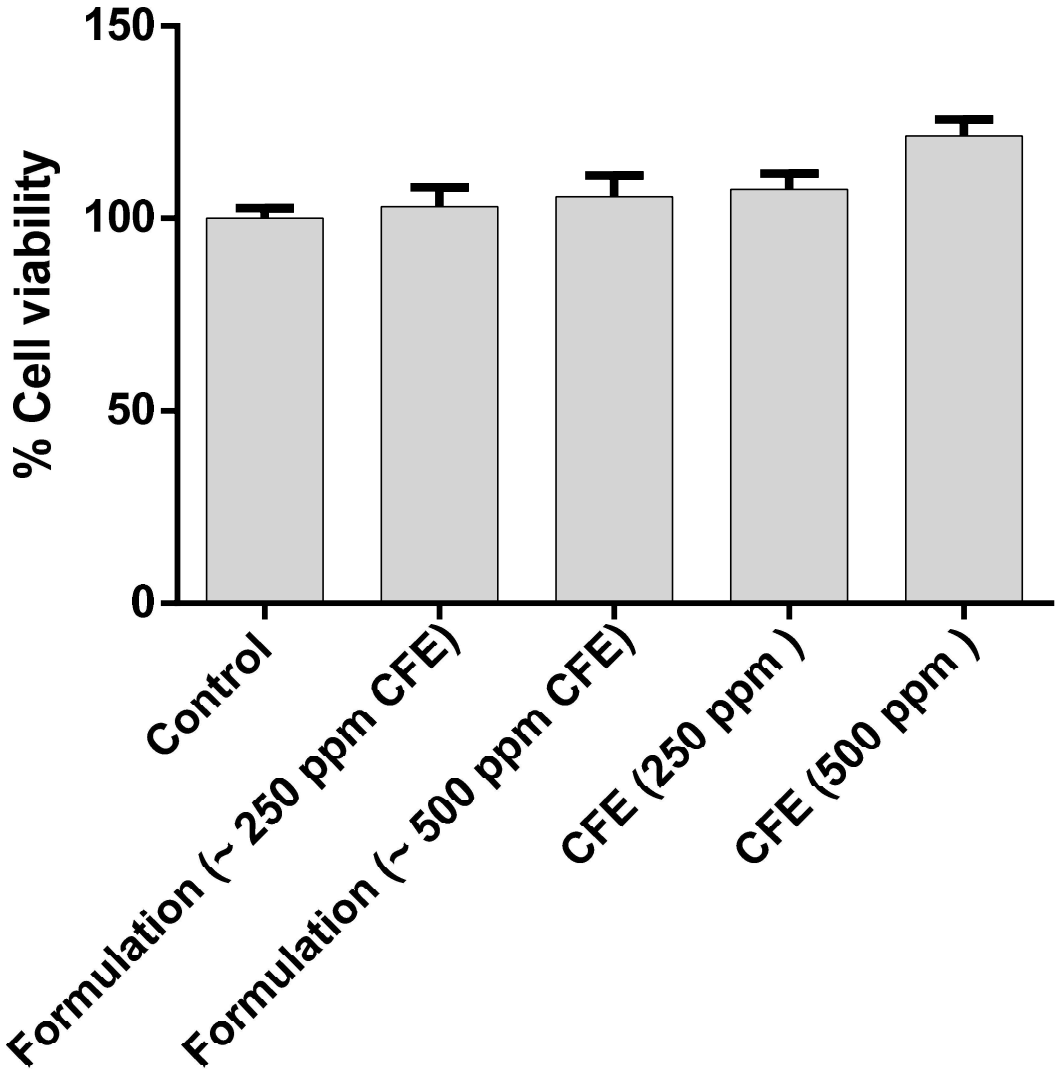

FIGURE 7.2: Result of *In-vitro* cell viability study with CFE and CFE loaded hydrogel sheet

TABLE 7.3: % Cell viability of collagen film with CFE and CFE in MTT assay

CFE loaded collagen film extract	Avg Absorbance at 570 nm (ref. filter of 655 nm) (±SD, n=8)	%Cell viability (±SD, n=8)
Only media (control)	0.2640 ±0.0307	100.0000% (±11.62887%)
10 µl (~50 ppm CFE)	0.2908 ±0.0189	110.9848 % (±7.1818 %)
25 µl (~125 ppm CFE)	0.3120 ± 0.0320	118.1818 % (±12.1212 %)
50 µl (~250 ppm CFE)	0.3146 ± 0.0462	119.1667 % (±17.4612 %)
100 µl (~500 ppm CFE)	0.3271 ± 0.0562	123.9015% (±21.2878 %)

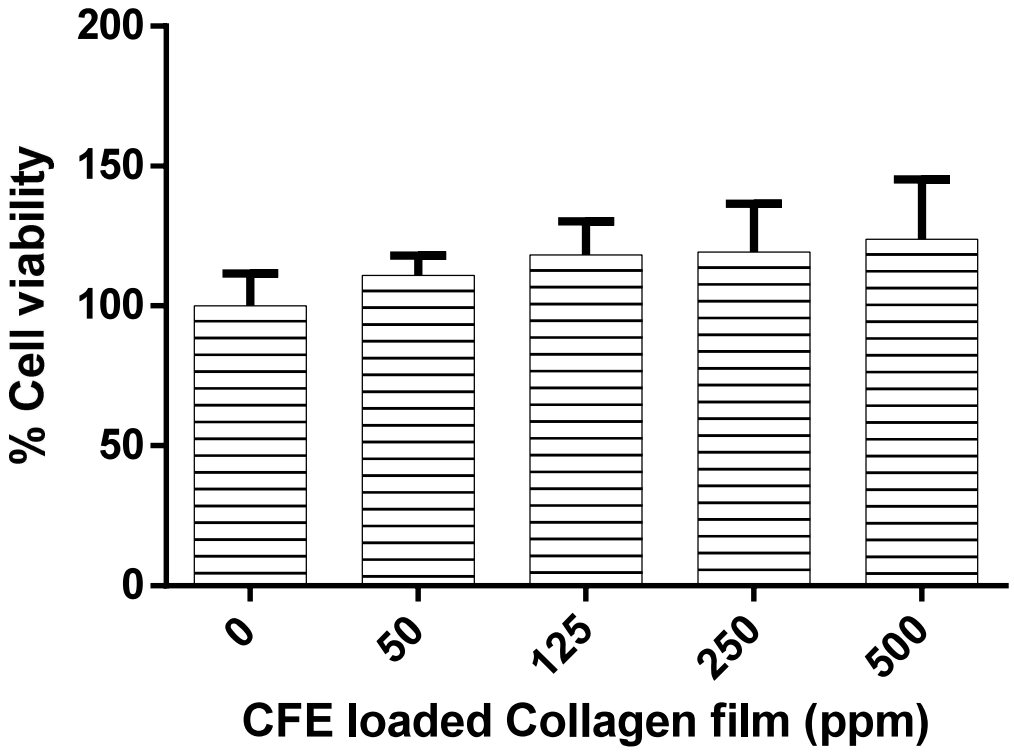

FIGURE 7.3: Result of i*n-vitro* cell viability study with CFE loaded collagen film

7.4.2 SCRATCH ASSAY: To evaluate the wound healing potential of CFE and prepared formulations, scratch assay was performed in which artificial injured cell (scratch) were treated and cell migration into the wound was observed in response to scratch. It is an attractive and cost effective *in-vitro* method which gives reliable and reproducible result to investigate new tissue formation during wound closure process (5). A single representative area is captured after the wounding (0 hr), after 24 hr and 48 hr incubation (fig. 7.4). The formulations and CFE treated well had more population of fibroblasts in the scratched area compared to only media treated and placebo formulations treated well. CFE increased the population of fibroblasts in the scratched area due to the migration of cells and/or by proliferation of the migrated cells (4). Active components of CFE mainly triterpenoids are responsible for the anti-inflammatory activity and also positively influence the wound healing effect by stimulating the proliferation and migration of fibroblasts (3). Fibroblasts, which are involved in granulation and collagen metabolism are stimulated by calendula extracts resulting in proliferation and migration within the wound site (6). Skin fibroblasts produce and organize the ECM of the dermis and communicate with each other and other cell types, playing an important role in regulating skin physiology (7-10) the scratch assay covers the second phase of wound healing characterized by proliferation and migration of either keratinocytes or fibroblasts (6, 11, 12). Cell migration and proliferation are very important steps during the second phase of wound healing.

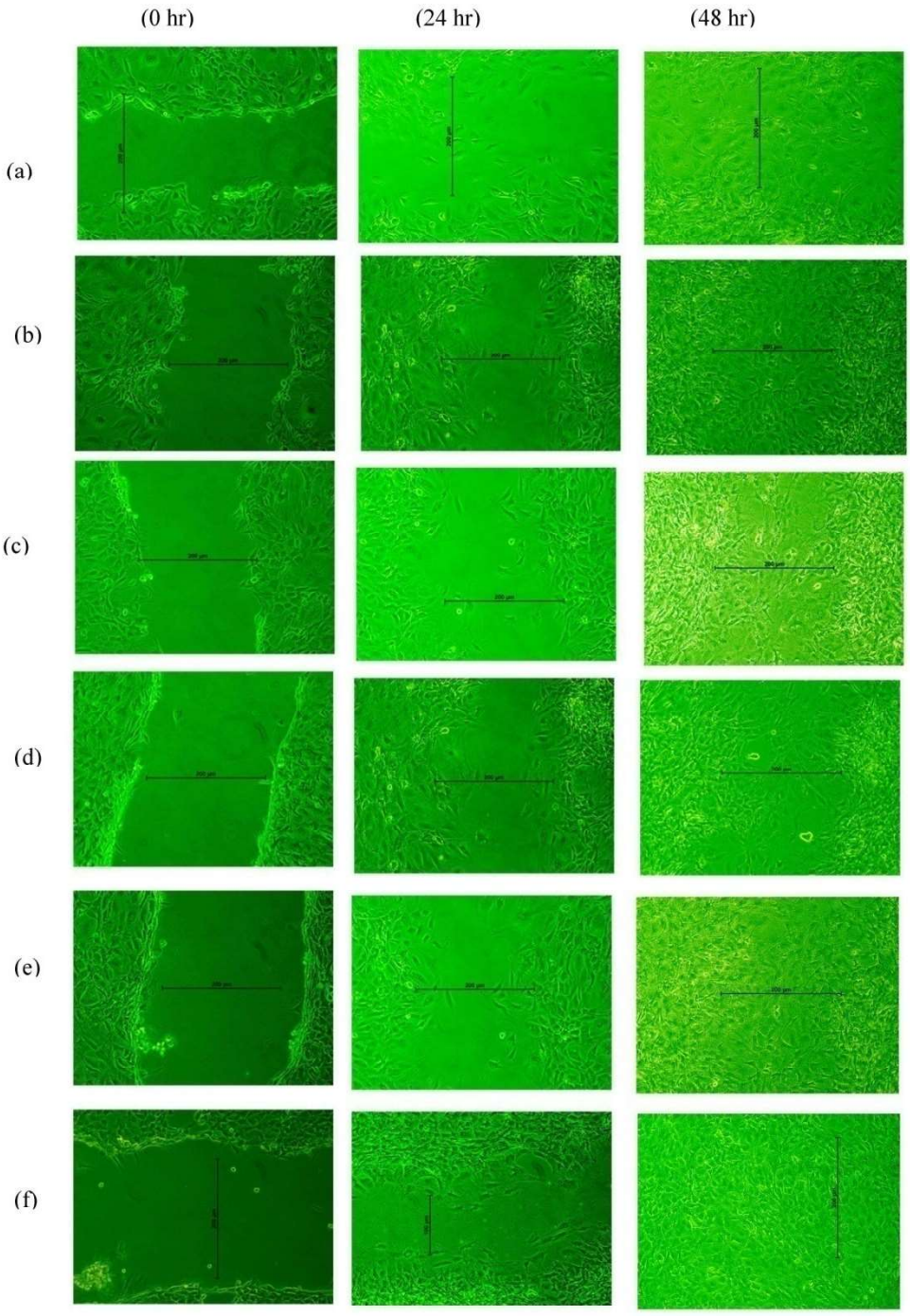

FIGURE 7.4: Single representative area of cell well after wounding (a) Only media (control), (b) CFE, (c) extract of collagen film without CFE, (d) extract of CFE loaded collagen film, (e) extract of hydrogel sheet with CFE and (f) extract of CFE loaded collagen film

7.5 CONCLUSION

- *Calendula* flower extract loaded hydrogel sheet and collagen sheet where found to be non-toxic to the fibroblasts. The result of scratch assay proved that CFE significantly promoted the migration and proliferation of cell leading to wound closure, confirming its usefulness for gaining first insight into the potential of formulation to repair injured dermis.

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