Evaluation of Corneal Wound Healing Activity of *Glycyrrhiza glabra* Ophthalmic Formulation by *In-vitro* Scratch Assay.

PURPOSE: To formulate a novel ophthalmic formulation with *Glycyrrhiza glabra*for improvingcorneal wound healingby *in vitro* scratch assay using Statens Seruminstitut Rabbit Corneal (SIRC) Cells.**METHODS**: In the present study, corneal wound healing activity was studied on statens seruminstitut rabbit corneal cell line. The method used to conduct the study was scratch assay method. The scratch was introduced into the confluent monolayer of cell line and treated with*Glycyrrhiza glabra* ophthalmic formulation. Evaluation of wound healing activity was compared with control group. Observations were noted at the beginning of study and at regular time intervals. Scratch measurement was observed. Quantitative parameters like percent wound closure, rate of cell migration, interlukin-6 concentration levels were measured. The study was performed according to protocols and standard operating procedures (SOP). All laboratory data has been accurately recorded and verified. **RESULT:**The result of this experiment obtained with statistical analysis of trial group and control group. Results were expressed as mean+/-SD and data analysed by one-way, two-way analysis of variance (ANOVA) followed by turkey's multiple comparison test. From the results it can be observed that *Glycyrrhiza glabra* ophthalmic formulation has shown statistically significant increase in percent wound closure ($p<0.05$) after completion of treatment as compared to control wells. Which indicated itswound healing potential in SIRC cell line by scratch assay method. **CONCLUSION:**Analysis of result concludes that *Glycyrrhiza glabra* ophthalmic formulation is proven as an ayurvedic alternative treatment modality for treatment of corneal wounds. *Glycyrrhiza glabra* ophthalmic formulation has shown statistically significant results, which is cost effective and preservative free. This formulation can be used for treating corneal wounds.

KEYWORDS: Corneal wound healing, *Glycyrrhiza glabra,*ophthalmic formulation, *In vitro*, Scratch assay.

1. INTRODUCTION

'Corneal blindness' is a major cause of visual impairment in developing countries like India and has been recognized as an important public health issue. This condition encompasses a range of eye conditions which alter the transparency of cornea, leading to corneal scarring and eventually blindness. Cornea is a thin, transparent, avascular membrane known as window to the eyes. It plays an important role in vision and immunological defence. Cornea being the anterior most structure of the eye; often affected by infections, trauma, chemical injuries, foreign body penetration, ultra-violet rays which leads to corneal wounds. Prolonged usage of

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contact lens, refractive error correction surgeries, pseudophakia induced bullous keratopathy, corneal abrasions hamper the optical performance.

The prevalence of corneal blindness in adult Indian population is 4.5 per 1000 (95%). According to the National Programme for Control of Blindness (NPCB); 1,20,000 people are corneal blind in the country.[1] The burden of corneal diseases in India is reflected by the fact that 90% of the global cases of ocular trauma and corneal ulceration. There is significant burden of corneal blindness in the adult Indian population.

Corneal wound healing plays a pivotal role in restoration of barrier and maintain integrity of cornea. Cell migration is the crucial step of wound healing. This process is well orchestrated with the involvement of several cell types. The stages of wound healing include- Haemostasis, inflammation, proliferation, migration and re-modelling.[2]For treating corneal wounds, topical instillation is considered as convenient and effective method.However, new eye drops which will exhibit anti-inflammatory effect along with faster wound healing are still desired in ophthalmic practice. This study aimed to formulate a novel ophthalmic formulation with *Glycyrrhiza glabra*to improve corneal wound healing. The characteristics of this ophthalmic formulation and its efficacy in re-epithelialization were evaluated.

The basic principle of two-dimensionalwound healing assay is destruction of confluent cellular monolayer, generating a cell-free region, which is available to cells for migration and repair.[3]Novel therapeutic approach to restore the corneal integrity and prevent secondary infection and visual loss can be achieved by prescribing ayurvedic medicines. Effective, convenient and inexpensive treatment modality should be used for corneal epithelial regeneration.

*Glycyrrhiza glabra*is a classical, widely used ayurvedic medicinal plant. Traditionally decoction of *Glycyrrhiza glabra*are used for treating cough, cold and wounds. Active constituents are Glycyrrhizin, triterpene saponin, glycyrrhetinic acid, aglycon of glycyrrhizin.[4]Since no elaborated scientific data were available concerning the corneal wound healing activity of *Glycyrrhiza glabra*, the present study was conducted.

2. MATERIAL AND METHODS

This experimental study for assessing corneal wound healing is divided into two sub types. First includes development of *Glycyrrhiza glabra* ophthalmic formulation and second consists of scratch assay experiment. Drug preparation done by standard operative procedure for extract preparation. Physicochemical analysis performed before instillation of drug in cells. A comprehensive general examination of cells performed. Corneal cell culture prepared. Scratch with pipette tip created in confluent monolayer. Observations made with help of phase contrast

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microscope. Parameters for quantitative assessment of corneal wound healing are rate of cell migration, wound closure, levels of interlukin-6 and percentage wound closure. Data analysed and results concluded at the end of study. All these parameters were assessed in three groups. Control, standard and trial group. Number of readings acquired and mean values with standard deviations were calculated. Quantitative representation in form of tables and graph assessed. Efficacy of prepared drug in comparison with standard silver nitrate compound studied and conclusion drawn. Further scope of this experiment analysed.

2.1 Development of *Glycyrrhiza glabra* ophthalmic formulation

I. Macroscopic and microscopic assessment of *Glycyrrhiza glabra*
II. Extract preparation procedure
III. Physicochemical analysis of Glycyrrhiza glabra ophthalmic formulation

2.2 Scratch assay experiment

a. Corneal cell culture preparation
b. Scratch wound assay
c. Incubation period
d. Instillation of medication
e. Observations based on parameters
f. Data acquisition by phase contrast microscope
g. Data analysis by computing software

2.3 Phytochemical assessment of *Glycyrrhiza glabra*

This test reveals presence of alkaloids, saponins, glycosides, carbohydrates, tannins and steroids. Aqueous and alcoholic extract are studied and results are compared. For testing carbohydrates benedict's test performed. Alkaloids tested with wagner's test and amino acid tested by ninhydrin test. Saponin by foam test, steroids by salkowaski reaction and taninns by potassium dichromate test which reveals negative result for aqueous extract and positive result for alcoholic extract.

2.4 Standardization of *Glycyrrhiza glabra*

Analytical tests were performed on dried sample of *Glycyrrhiza glabra.* Debris in sample were cleaned. Parametric observations are mentioned in the table below.

Table 1: parametric observations of *Glycyrrhiza glabra*

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Parameters	Observation	Findings
Colour	Yellowish brown	Complies
Odour	Characteristic	Complies
Taste	Sweetish	Complies
Weight	50 gm	NA
pH	6.13	NA
Loss on drying	5.65 %	NA
Total Ash	0.11 %	NMT 10 %
Acid insoluble ash	0.08 %	NMT 2.5 %
Water soluble extractive	33.80 %	NLT 20 %
Alcohol soluble extractive	14.69 %	NLT 10 %

Figure 1: *Glycyrrhiza glabra* powder

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2.5 Aqueous extract preparation of *Glycyrrhiza glabra*

- 50 gm dry powder of *Glycyrrhiza glabra*was weighed with help of electronic weighing machine.
- Debris were removed from sample, was soaked in water overnight and filtered.
- Soxhlet apparatus was assembled with aseptic precautions under sterile condition.
- Distilled water taken in measuring cylinder for aqueous extract preparation.
- Soxhlet extractor fixed into round bottom flask and it was placed over heating mantle.
- Condenser fixated,in-let and out let pipes attached for water supply.
- Temperature of heating mantle maintained between 70 to 90 degree celsius.
- 250 ml of filtered solution of yashtimadhu kept for heating in soxhlet apparatus.
- Condensation of vapours occurred and collected drop by drop.
- Clear, transparent solution of *Glycyrrhiza glabra*obtained.
- Final extraction filtered through Whatman's filter paper.
- Extract collected in transparent sterile container and stored at 4 degree celsius.

Figure 2: Filtered sample of *Glycyrrhizaglabra*

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Figure 3: Aqueous extraction by soxhlet apparatus

Table 2: analysis of standardization parameters of *Glycyrrhiza glabra* extract

Parameters	**Findings**
Sample name	*Glycyrrhiza glabra* extract
Sample quantity	50 ml
Colour	Clear, colourless
Taste	Slightly bitter
Odour	Aromatic
Rancidity	No pink discolouration observed
pH of aqueous solution (10%)	7.10
Specific gravity	0.97 gm/ml
Refractive index	1.3898
Density	0.98 gm/ml
Viscosity	6.74 Pa.s

2.6 Experimental procedure of scratch assay[5]

Test sample was filtered before using for study. Scratchassay performed based on protocol. Chemicals and materials used are mentioned as follows:

Chemicals and materials

- Cell culture plates – 12 well plates (Abdos)
- Cell culture flask – T25 flask (Abdos)
- Trypsin/ EDTA – 0.25% trypsin and 0.02% EDTA in Dulbecco's phosphate buffered saline

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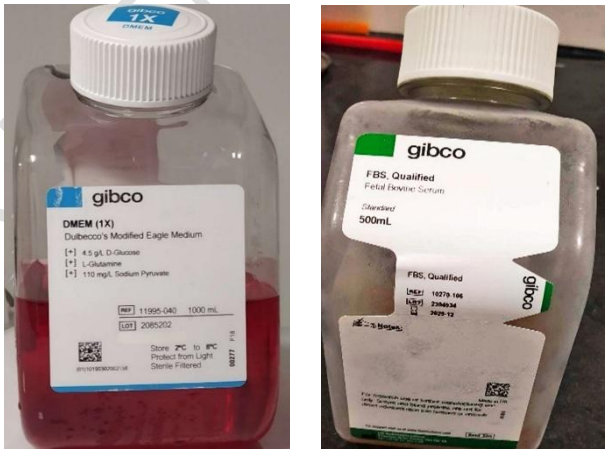
- DMSO – dimethyl sulfoxide
- Cell culture medium – Eagle's minimum essential medium (MEM) containing 10% foetal bovine serum.
- Cell line – SIRC (statens seruminstitut rabbit cornea)
- Culture conditions – 37 degree celsius with 5% Co_2

Reagents

- Dulbecco's phosphate buffered saline (Himedia)
- 0.25% trypsin
- 0.02% EDTA
- Dimethyl sulfoxide (DMSO)
- 1 mg ml^{-1} poly-L-lysine
- Phosphate buffered saline (PBS)
- Eagle's minimum essential medium
- 10% foetal bovine serum

Figure 4:Dulbecco's modified eagle medium (DMEM) and Foetal bovine serum (FBS)



Equipment:

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- 60 mm sized 12 well plates (Abdos tissue culture dishes)
- T25 flasks (Abdos)
- Sharpie's marker
- P200 pipet tip
- Hemo cytometer
- Co_2 supply (5%)
- Phase contrast microscope
- Stage incubator
- Camera with image analysis software

Preparation of cells

SIRC (statens seruminstitut rabbit cornea) epithelial cells were cultured in Eagle's minimum essential medium supplemented with 10% foetal bovine serum.Cells were maintained in a humidified atmosphere of 5% Co_2 at 37 degree celsius. Cells were seeded in 12 well plates at a concentration of 1,00,000 cells per well. The plates were incubated at 37 degree celsius and 5% Co_2 atmosphere till confluent monolayer was obtained.

Coating of cell culture

60 mm dishes with proper ECM substrates were incubated overnight at 4 degree celsius for 2 hours at 37 degree celsius without shaking the tissue culture plate. Unbound ECM substrate was removed and dish coated with bovine serum albumin for one hour at 37 degree celsius. Culture well dishes then washed with PBS. 3 to 5 ml media used before plating the cells. Amount of serum in medium appropriate for *in vitro* scratch assay is identified. Low percentage of serum used to minimize the proliferation of cells.

Passaging of cell culture

Confluent cells grow in tissue culture are washed with PBS twice and mixed with medium containing serum. Pipetting of solution done and cells dispersed evenly. Aliquot taken from cell suspension. Determination of cell count with the help of hemo cytometer performed. Cells prepared are plated on 60 mm dishes to create confluent cell monolayer. Dishesincubated for 6 hours at 37 degree celsius. Adherent cells are spread on substrate.

Protocol of scratch wound assay

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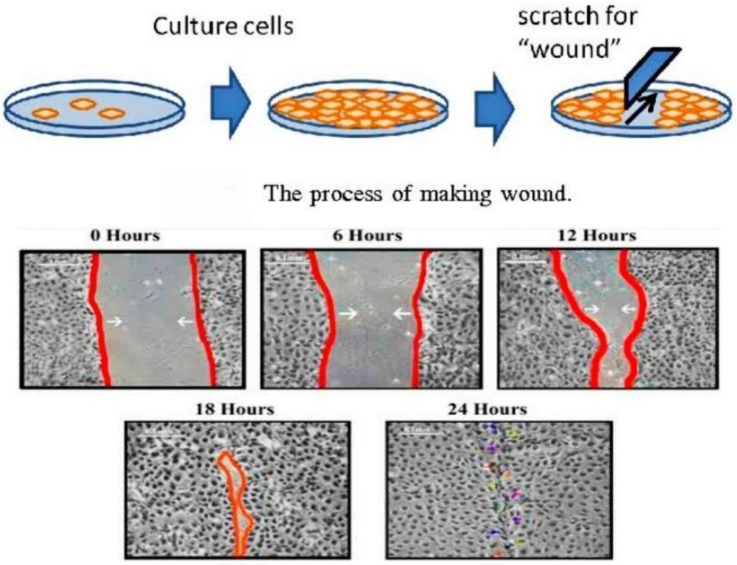
a. Cell culture preparation
b. Scratch wound assay
c. Data acquisition
d. Data analysis

Figure 5: Scratch assay



3. OBSERVATIONS AND RESULT

3.1Assessment criteria

1. Images observed at regular time intervals with phase contrast microscope
2. Scratch measurement in um
3. Wound closure in um
4. Percent wound closure at beginning and end of study
5. Rate of cell migration
6. Concentration of interleukin-6 in pg/ml

3.2Observations

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Table 3: Scratch measurement (um)

Group	**0hour**	**24hours**	**48hours**
Control	69.33	64.15	59.09
Standard	68.78	55.79	42.28
Glycyrrhizaglabra ophthalmic formulation	68.42	56.23	40.15

Graph 1: Scratch measurement

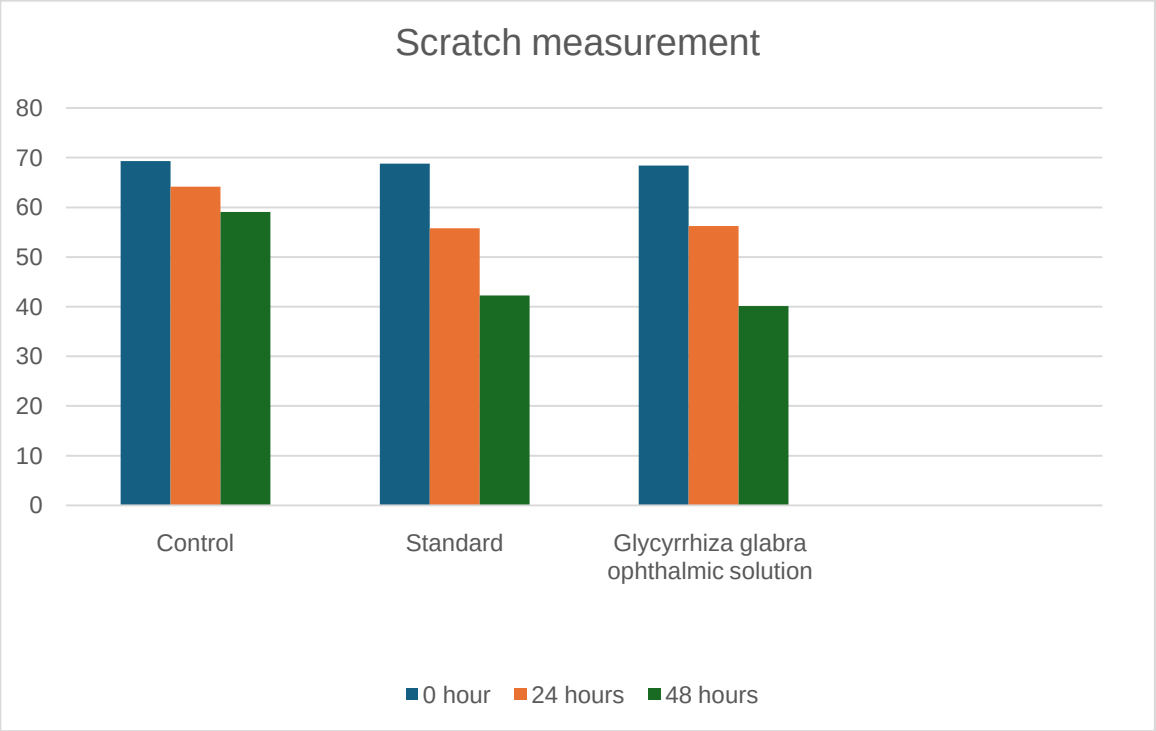

Table 4: Wound closure (um)

Group	**24 hr – 0 hr**	**48 hr – 24hr**
Control	5.17	5.07
Standard	13.00	13.51
Glycyrrhizaglabra ophthalmic formulation	13.68	13.52

Graph 2: Wound closure

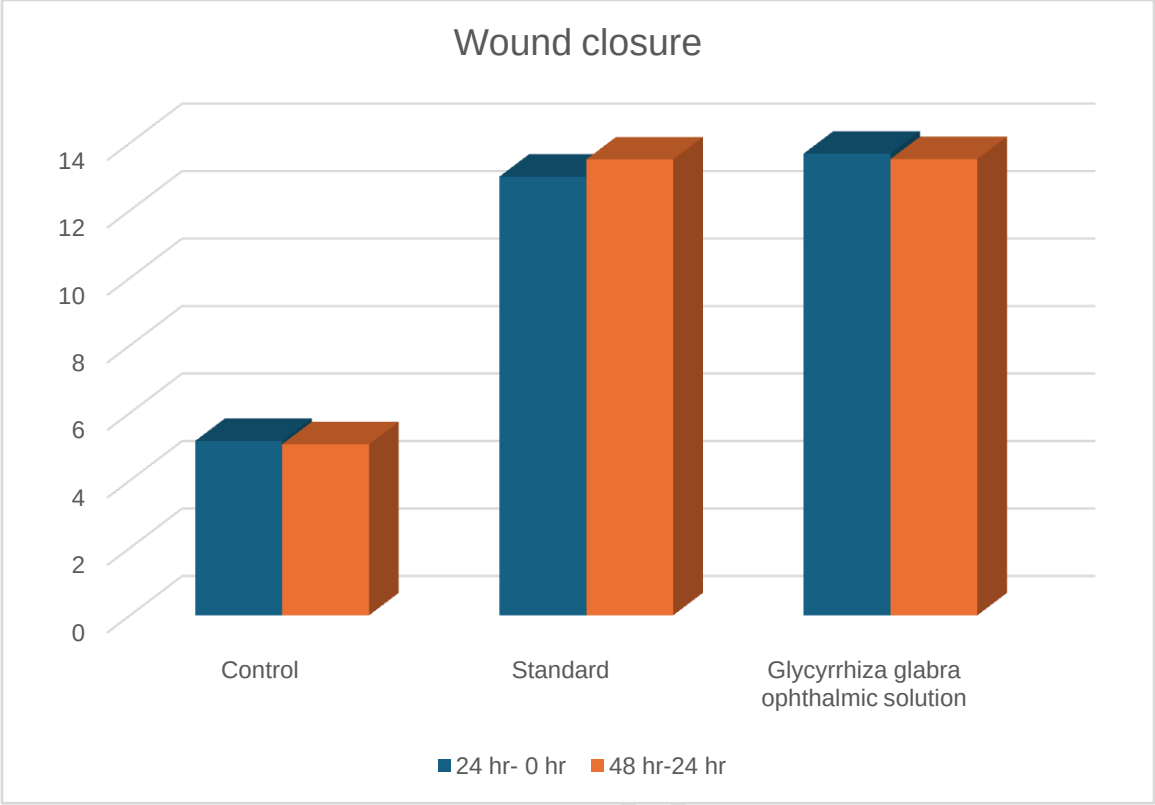

Table 5: Percent wound closure (%)

Group	**24 hr**	**48 hr**
Control	7.39	14.58
Standard	18.76	38.69
Glycyrrhizaglabra ophthalmic formulation	20.25	39.05

Graph 3: Percent wound closure

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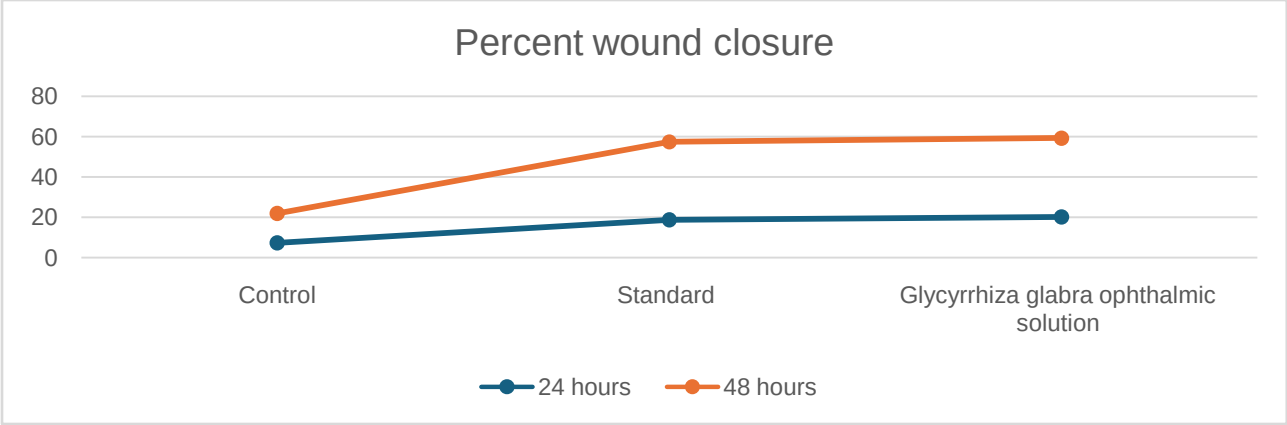


Table 6: Rate of cell migration

Group	**24 hr**	**48 hr**
Control	0.22	0.21
Standard	0.52	0.55
Glycyrrhizaglabra ophthalmic formulation	0.57	0.54

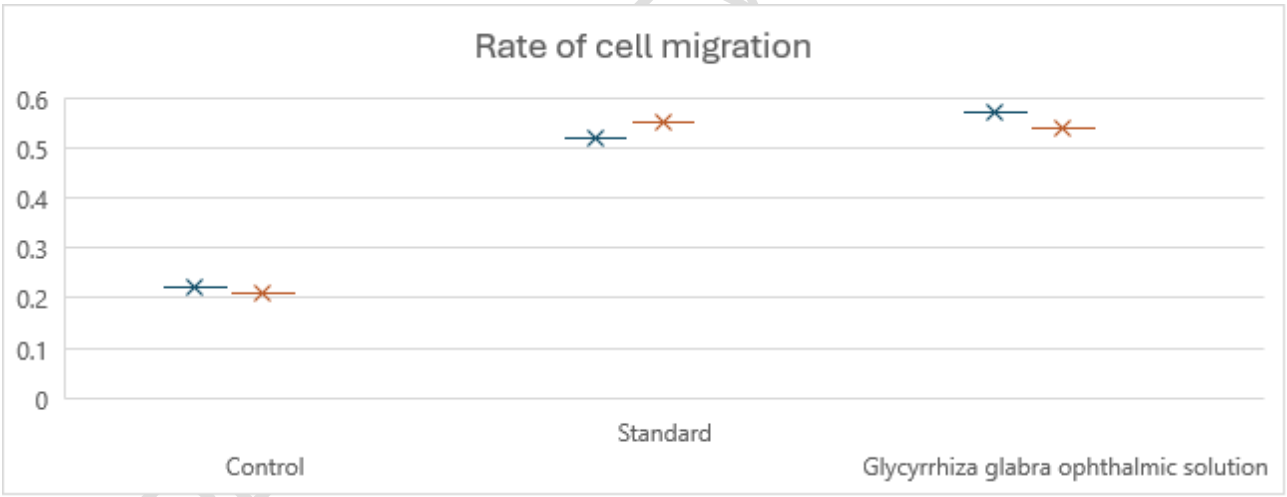
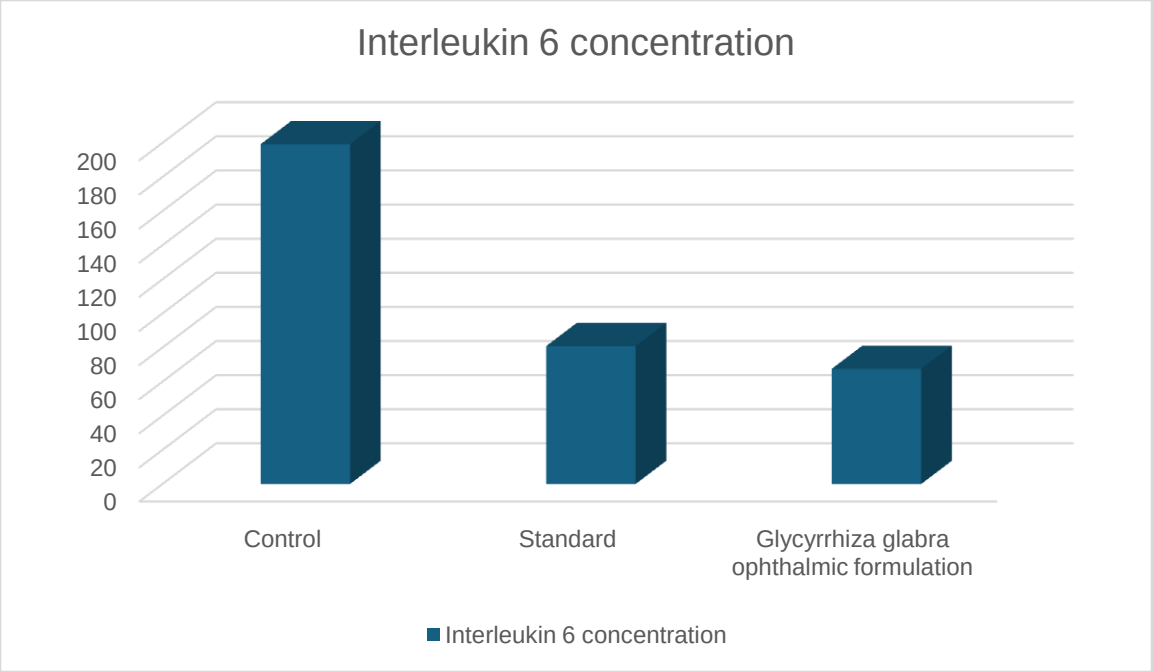


Table 7: Interleukin 6 levels

Group	**Interleukin6concentration** (pg/ml)
Control	199.17
Standard	80.83
Glycyrrhizaglabra ophthalmic formulation	67.50



3.3 Statistical analysis

Graph pad prism version 8.00 was used for statistical analysis.Statistical significance was considered at $p<0.05$, $p<0.01$ and $p<0.001$ as compared to control in all cases. The extracted sheets of statistical analysis are provided along with the report in txt format.

3.4Result

The result of this experiment obtained with statistical analysis of trial group and control group. Results were expressed as mean+/-SD and data analysed by one-way, two-way analysis of variance (ANOVA) followed by turkey's multiple comparison test. From the results it can be observed that *Glycyrrhiza glabra* ophthalmic formulation has shown statistically significant increase in percent wound closure ($p<0.05$) after completion of treatment as compared to control wells. Which indicated its wound healing potential in SIRC cell line by scratch assay method.

4. Potential Pitfalls and Trouble Shooting

- Duration of study was shorter; study should be carried out on larger sample group for longer duration.
- Study performed was *in vitro*, further *in vivo* and clinical trials required.
- Advanced technological support for imaging and quantitative analysis can be taken for obtaining better results.

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- Cell culture models having similar cellular composition as human cornea like clonetics, can be used for studying transepithelial permeability.

5. Conclusion

Analysis of result concludes that *Glycyrrhiza glabra* ophthalmic formulation is proven as an ayurvedic alternative treatment modality for treatment of corneal wounds. *Glycyrrhiza glabra* ophthalmic formulation has shown statistically significant results, which is cost effective and preservative free. This formulation can be used for treating corneal wounds.

6. Ethics statement

The present study was reviewed and approved by Institutional Ethics Committee of Dr. D. Y. Patil College of Ayurved and Research Centre, Maharashtra, India. (Number)

7. Author contributions

SV and PJ made substantial contributions to conception, design and interpretation of data. SV wrote initial draft of the manuscript. PJ and MV reviewed the manuscript critically for important intellectual content and gave final approval of the version to be submitted.

8. Funding

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9. Conflict of interest

All authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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