8.1 INTRODUCTION

For evaluating the eye irritation potential of raw materials and formulations, *in vivo* Draize test has been employed from many years.

But in recent years, interest in animal alternative tests has been increased and various methods have been developed and evaluated for *in vitro* eye irritation tests such as cell-based cytotoxicity methods, reconstitute tissue models, organotypic methods, chorio-allantoic membrane (CAM) methods, isolated organ methods etc as an alternative to Draize test in an attempt to reduce or replace *in vivo* studies with alternative studies (Takahashi et al., 2008).

Among these methods, cell line based cytotoxicity methods are widely used for evaluating ocular irritation studies because they are cost-effective and sensitive assays, often easy to perform, repeat, manipulate, and score (Borenfreund and Borrero, 1984; Huhtala et al., 2002).

8.2 CELL LINE AND MATERIALS

SIRC cell line was purchased from National Centre for Cell Sciences (NCCS), Pune, India. Minimum Essential Medium Eagle Medium (MEM), N-[2- hydroxyethyl] piperazine- NV-[2-ethanesulphonic acid] (HEPES), 10% Fetal Bovine Serum (FBS), penicillin/streptomycin, Trypsin-EDTA and 96 well plates were purchased from Himedia, Mumbai, India. Methyl thiazoly diphenyl-tetrazolium bromide (MTT) was purchased from Sigma Aldrich (St. Louis, MO, USA).

8.3 EQUIPMENTS

CO_2 Incubator (Jouan IGO 150 Cell Life CO_2 Incubator, Thermo Fisher Scientific, Mumbai, India)

ELISA microplate reader ((Biorad, Model 680 XR, Mumbai, India)

Laminar air flow system (Swastika Electric and Scientific Works Ltd. Ambala, India)

CKX41 Inverted Microscope (Olympus, USA)

8.4 METHODS

8.4.1 Short Time Exposure Test and category classification

For the study, SIRC cells ($7x10^3$ / well) in 96-well plates were exposed to 200 µl of 0.05, 0.5, 5% (w/v) test chemical solutions (DPPC, GMS, CS HCl, HA, TPP, SPC, GCV and TA) prepared by dissolving them either in physiological saline or saline with 5% (w/v) DMSO , according to solubility of excipients, for 5 min

The ratio (%) of MTT formazan absorbance for each test chemical to the absorbance of MTT formazan for control represented cell viability (triplicate determinations) using the following formula:

$$\%\ Cell\ Viability = \frac{ABS\ Samples}{ABS\ Control}\ x\ 100$$

The control group cells were exposed to physiological saline and saline with 5% DMSO. After obtaining cell viability in the STE test, which used a 5 min exposure to test chemicals in physiological saline, saline with 5% DMSO, category and rank classifications were determined for each test concentration. A concentration of test chemical that had a CV of 70% or less was categorized as an irritant (I) and a concentration of test material that had a CV greater than 70% was categorized as a non-irritant (NI).

The cell viability of STE test data for each chemical was scored in order to estimate a rank categorization for eye irritation potential.

Fig Error! No text of specified style in document.**.1 Schematic representation of rank classification for short term exposure test**

Classification of general eye irritation potential for the chemicals was based on the added scores of 1, 2, or 3 for the STE test. A rank of 1 corresponded to a chemical being categorized as a minimal ocular irritant while a rank of 2 categorized a chemical as a moderate ocular irritant and a rank of 3 categorized a chemical as a severe ocular irritant. Rank 1: minimally irritant; Rank 2: moderate irritant; Rank 3: severe irritant.

8.4.2 *In vitro* cytotoxicity assay

A promising nanoparticulate carrier system intended for ocular use must be capable of delivering sufficient levels of the active agent without compromising the viability of the host cells (Aksungur et al., 2011). The cytotoxicity study on drug loaded formulations (GCV emulsomes, TA emulsomes, GCV NPs and TA NPs) was done for 24 hours, in order to study the effect of different concentrations on SIRC cells.

The percentage of cell viability was expressed as the percentage calculated by the following equation:

$$\%\ Cell\ Viability = \frac{ABS\ Samples}{ABS\ Control}\ x\ 100$$

Where, ABS samples represent the absorbance values of those wells exposed to the formulations and ABS control was the absorbance values of those wells treated with medium.

8.5 RESULTS AND DISCUSSION

8.5.1 Ganciclovir loaded Emulsomes

8.5.1.1 Short term exposure test

The effect of plain GCV solution and excipients (DPPC and GMS) used in the preparation of GCV emulsomes at low (0.05 % (w/v)), medium (0.5 % (w/v)) and high (5 % (w/v)) concentrations were studied on the viability of SIRC cell lines.

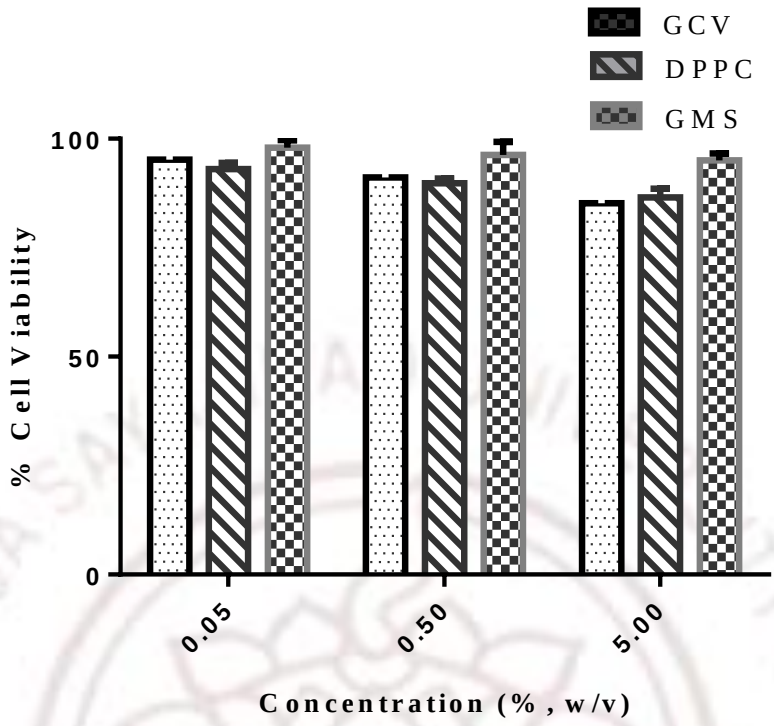

Fig Error! No text of specified style in document.**.2 Graph showing % cell viability of excipients used in GCV emulsomes preparation at 0.05, 0.5 and 5 % (w/v) concentration**

This indicate that all the excipients chosen for the preparation of emulsomes, in the concentration range 0.05 %, 0.5 and 5 % (w/w) were minimal irritant and non toxic to rabbit SIRC cell line, following 5 min exposure time.

8.5.1.2 *In vitro* cytotoxicity assay

In order to determine the effect of increasing concentration of GCV loaded emulsomes on the viability of SIRC cells, cytotoxicity assay was performed for 24 hrs. There was slight decrease in cell viability with increasing concentrations of GCV loaded emulsomes (Fig. 8.3).

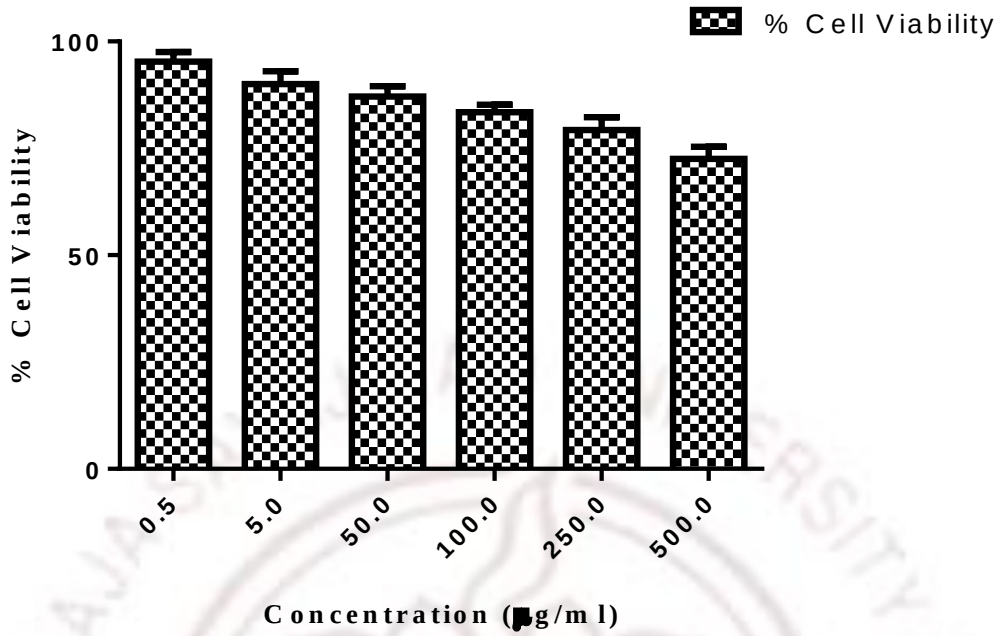

Fig Error! No text of specified style in document.**.3 Graph showing effect of different concentration of GCV emulsomes on % cell viability of SIRC cells**

8.5.2 Triamcinolone acetonide loaded emulsomes

8.5.2.1 Short term exposure test for TA loaded emulsomes

The effect of drug (TA) and excipients (DPPC and GMS) used in the preparation of TA emulsomes, at low (0.05 % (w/v)), medium (0.5 % (w/v)) and high (5 % (w/v)) concentrations were studied on the viability of SIRC cell lines.

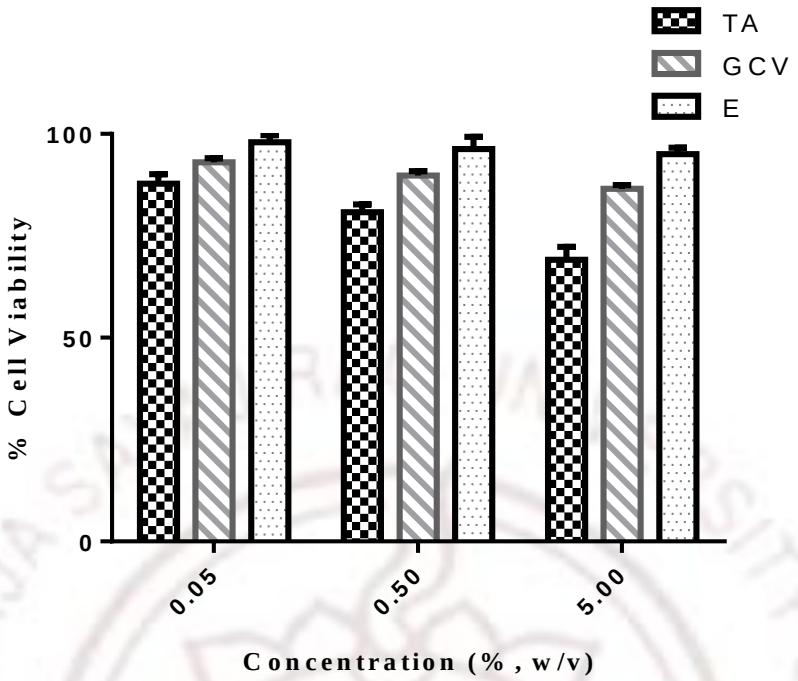

Fig Error! No text of specified style in document.**.4 Graph showing % cell viability of excipients used in TA emulsomes preparation at 0.05, 0.5 and 5 % (w/v) concentration**

This indicate that the excipients choosen for the preparation of emulsomes, in the concentration range 0.05 %, 0.5 and 5 % (w/w) were minimal irritant and non toxic to rabbit SIRC cell line, following 5 min exposure time.

In contrast, when cells were treated with free TA at equivalent concentration, % cell viability dropped from 87.78 ± 2.31 % to 69.12 ± 3.18 % at 0.05 and 5% (w/v) concentrations, respectively. Earlier experimental and clinical work done by researchers on commercial product of TA (Kenacort- A) also showed that the product was toxic to lens and retina (Retinal Pigmented Epithelial cells) because of the presence of benzyl alcohol, as vehicle. Moreover, there were concerns about cytotoxicity caused by the crystalline form of aggregated TA, as well (Szurman et al., 2006; Narayanan et al., 2006).

8.5.2.2 *In vitro* cytotoxicity assay for TA loaded emulsomes

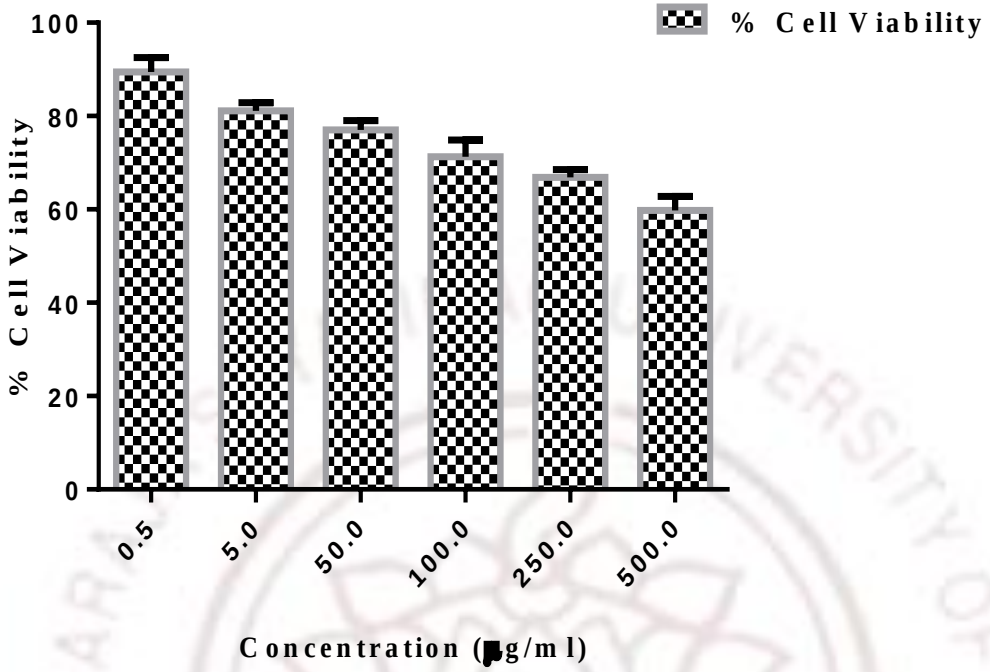

Fig Error! No text of specified style in document.**.5 Graph showing effect of different concentration of TA emulsomes on % cell viability of SIRC cells**

In order to determine the effect of increasing concentration of TA loaded emulsomes on the viability of SIRC cells, cytotoxicity assay was performed for 24 hrs..

8.5.3 Ganciclovir loaded nanoparticles

8.5.3.1 Short term exposure test for GCV loaded nanoparticles

One of the major requirements for the cationic polymeric nanoparticles used for drug delivery is their low cytotoxicity, as cationic polymers are known to exhibit cytotoxic effect by inducing cell membrane damage (Aksungnr et al., 2011Therefore, in this study, the effect of different excipients including CS HCl (CS HCl, HA and TPP) used in the preparation of GCV nanoparticles was investigated at low (0.05 % (w/v)), medium (0.5 % (w/v)) and high (5 % (w/v)) concentrations on the viability of SIRC cells.

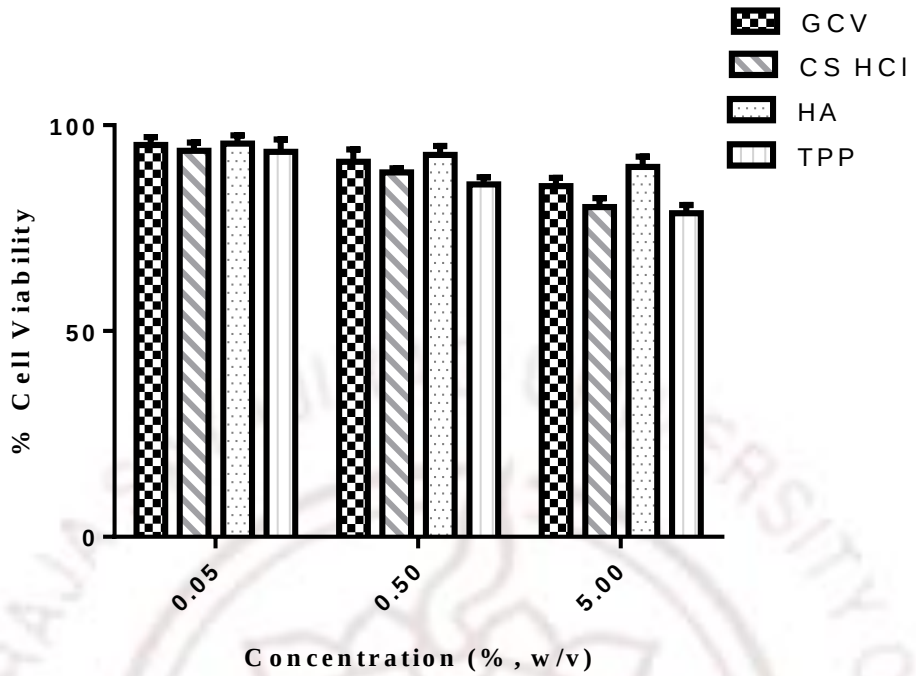

Fig Error! No text of specified style in document.**.6 Graph showing % cell viability of excipients used in GCV nanoparticles preparation at 0.05, 0.5 and 5 % (w/v) concentration**

This indicate that all the excipients choosen for the preparation of nanoparticles, in the concentration range 0.05 %, 0.5 and 5 % (w/v) were minimal irritant and non toxic to *In vitro* cytotoxicity assay for GCV loaded nanoparticles

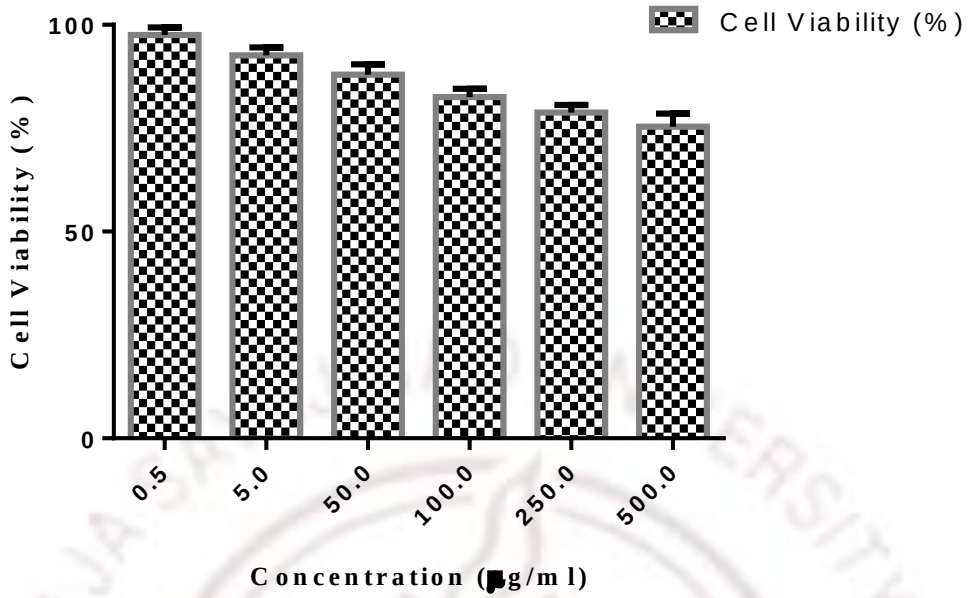

Fig Error! No text of specified style in document.**.7 Graph showing effect of different concentration of GCV nanoparticles on % cell viability of SIRC cells**

The cell toxicity of GCV loaded nanoparticles was investigated by studying the dose-dependent effects on the cell viability of SIRC cells for 24 hrs. Cell viability was higher when the concentration of nanoparticulate system was lower (Fig. 8.7).

8.5.4 Triamcinolone acetonide loaded nanoparticles

8.5.4.1 Short term exposure test for TA loaded nanoparticles

The effect of drug and different excipients (TA, CS HCl and Lecithin S 100) used in the preparation of TA nanoparticles at low (0.05 % (w/v)), medium (0.5 % (w/v)) and high (5 % (w/v)) concentrations were studied on the viability of SIRC cell lines.

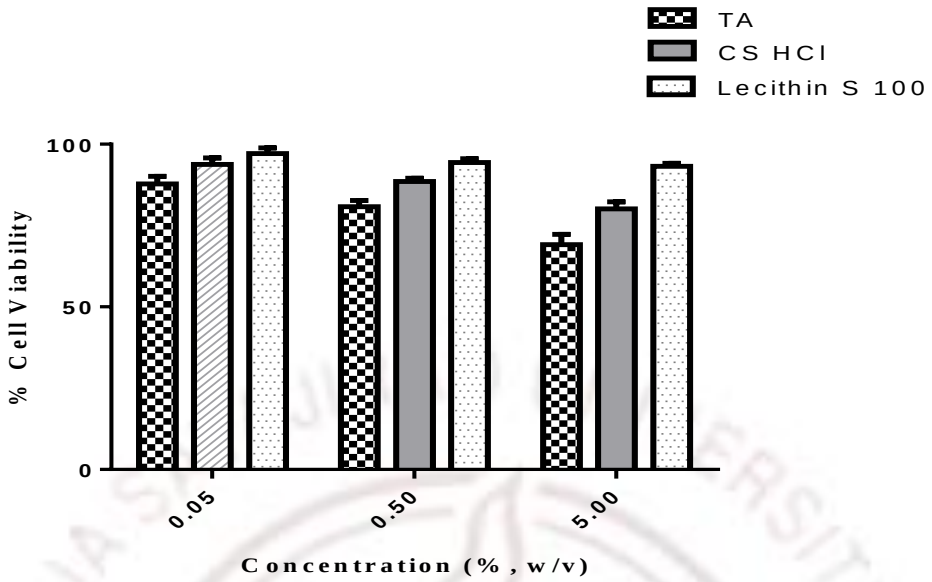

Fig Error! No text of specified style in document.**.8 Graph showing % cell viability of excipients used in TA nanoparticles preparation at 0.05, 0.5 and 5 % (w/v) concentration**

8.5.4.2 *In vitro* cytotoxicity assay for TA loaded nanoparticles

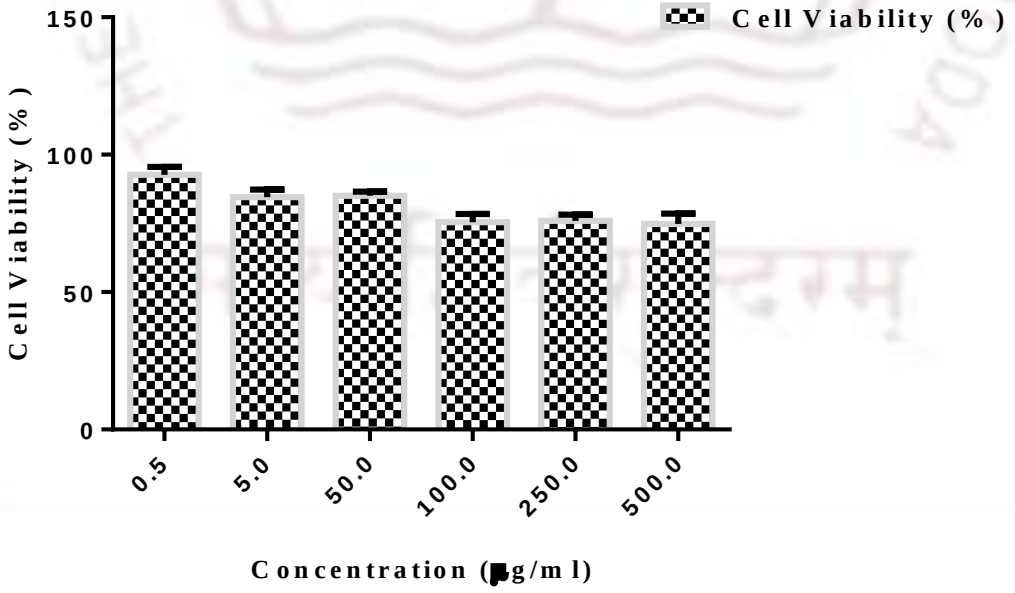

Fig Error! No text of specified style in document.**.9 Graph showing effect of different concentration of TA nanoparticles on % cell viability of SIRC cells**

These results showed that the TA loaded nanoparticles reduced the cytotoxicity of TA to SIRC cells as compared to that of TA suspension which could be because of the encapsulation of TA in nanoparticles (Fig. 8.9).

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