5.1. *Ex vivo* permeation studies

*Ex vivo* permeation of silymarin from NLC 9 gel was evaluated by using full thickness abdominal skin, which was excised from adult wistar rats weighing 160–200 g of age 15-18 weeks. Visceral side of the freshly excised skin was cleaned by removing adhering subcutaneous tissue with a scalpel. From the skin epidermal hair was removed and skin hydrated for 24 hr. in phosphate buffer solution (PBS) at pH 7.4. The skin samples were mounted on Franz diffusion cell and the receptor chamber filled with 28 ml diffusion medium. Diffusion medium contained Phosphate buffer solution of pH 7.4 containing 0.5 %v/v of Tween 60 and wasstirred continuously by using a magnetic stirrer. The skin was placed on the receptor chamber with the stratum corneum facing upward in the receptor chamber and then the donor chamber was clamped. The excess skin was trimmed and the whole assembly was put on a magnetic stirrer to continuously stir the medium present in the receptor compartment. The diffusion cell was placed in the diffusion assembly to stabilize at 37°C On the epidermal side of the skin, 1 gm of the silymarin-NLC gel and commercial formulation each were applied on the exposed surface area of 0.64 cm² respectively. Receptor phase was stirred constantly throughout the experiment and the receptor medium was water jacketed at 37°. At set intervals of 0, 1, 2, 5, 7, 9, 12 and 24 hr respectively, 2.0 ml of the receptor phase was removed and immediately replaced by an equal volume of PBS (pH 7.4) solution. Samples were analysed spectrophotometrically at λ<sub>max</sub> of 281 nm, in triplicate, for determination of the content of silymarin. The quantity of silymarin diffused per unit area (Q/A) versus time (hr) was plotted. The steady-state flux (J<sub>ss</sub>) of silymarin was calculated from the slope of the plot using linear regression analysis (Saupe et al 2015) (Bhaskar et al 2009). Permeability coefficient (K<sub>p</sub>) of silymarin through the membrane was calculated through the following equation:

\[ K_p = \frac{J_{ss}}{C} \]

(where C is initial concentration of dug in the donor compartment)
5.2. Cell proliferation activity by sulfo rhodamine-B (SRB) assay

5.2.1. SK-MEL-2 Cell line study for Silymarin-NLC gel

Anti-cell proliferation activity of silymarin-NLC 9 gel (test), Phytosome marketed formulation and positive control were assessed by the SRB assay on human melanoma cell line (SK-MEL-2). All the three samples were diluted by Dulbecco’s modified eagle medium (DMEM), supplemented with 2% w/v inactivated foetal bovine serum, to acquire a stock solution of 5 mg/mL concentration, which was filtered and centrifuged. Serial dilutions of 10, 20, 40, 80 µg/mL were made from the stock solution. Approximately 0.1 mL of the diluted cell suspension which contained about 10,000 cells was added to every well of the 96-well plate. After 24 hr, supernatant was flicked off, the monolayer washed with the medium and 100 µL of various concentrations of test drug added. Subsequently, plates were incubated at 37°C for 3 days. The cells were fixed using cold trichloroacetic acid (TCA) for 1 hr at 4°C and then washed with distilled water to remove excess TCA, and allowed to dry in air. After 72 hr, drug solutions present in wells was discarded and 50 µL of SRB solution was added to every well and allowed to stain for 30 minutes. The plate was washed with 1% v/v acetic acid to remove unbound dye and then allowed to dry in air. About 100 µL of 10 mM Tris buffer base (pH 10.5) was added to each well and the plates were gently shaken for 05 minutes on a shaker to extract the bound SRB. The absorbance was measured in triplicate by using a microplate reader (ELx800, Bio-Tek) (Vichal et al 2006). Inhibitory concentration 50 (IC 50) values represent the sample concentrations required to inhibit 50% of cell proliferation and were calculated from a calibration curve by linear regression using Microsoft Excel. All experiments were performed in triplicate (Sebaugh 2011).

5.2.2. SK-MEL-2 Cell line study for Silymarin-5-Fluorouracil NLC gel

Anti-cell proliferation activity of Silymarin-5-Fluorouracil NLC gel (test) and positive control was assessed by the SRB assay on human melanoma cell line (SK-MEL-2). 5-Fluorouracil has broad spectrum of activity with a broad spectrum of activity against tumours alone or in combination with other agent. The mechanism behind the cytotoxicity and cell death activity of 5-FU is its interference with nucleoside metabolism in RNA and DNA (Midena et al 2000) (Goette 1981). Experimental method was followed as explained earlier.

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In vivo studies

5.3. Skin Irritation Study

Animals: The protocol for the experimentation, transportation and care of the animals used in study was approved by Institutional Animal Ethical Committee (BBDNIIT/IAEC/057/2014) and the handling was done as per CPCSEA guidelines.

Method: For the skin irritation study of the NLC 9 gel formulation, the rats were divided into two groups with each group comprising three rats. Hair was depleted from the back of the rats with the help of depilatories and the area was specked on both sides. One side served as the control while the other side as a test. NLC gel (500mg/rat) was applied once a day for seven days and the skin irritation from the formulation was determined by observing the skin for possible sensitivity and skin reactions such as edema, rash and redness.

Observation: The reactions, defined as erythema and edema, were evaluated according to the scoring system for skin reactions (Table 37)(Majumder et al 2015) (Joshi and Patravale 2008) (Liu et al 2007).

Table 37: Scoring system for skin reaction

<table>
<thead>
<tr>
<th>Reactions</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Erythema</td>
<td>No Edema</td>
</tr>
<tr>
<td>Very slight erythema</td>
<td>Very slight edema</td>
</tr>
<tr>
<td>Slight patchy erythema</td>
<td>Well defined edema</td>
</tr>
<tr>
<td>Moderate erythema</td>
<td>Moderate edema (about 1mm)</td>
</tr>
<tr>
<td>Severe erythema with redness</td>
<td>Severe edema (more than 1mm)</td>
</tr>
</tbody>
</table>
5.4. UVInduced Skin edema study

5.4.1. Animals and experimental method

Male Wistar rats, having a body weight between 160 and 190 g, were randomly divided into three groups of six rats each, where every group received different topical treatment. The animals were housed at constant temperature and humidity levels and given water *ad libitum*. One group was treated with NLC 9 silymarin gel (containing 900 μg silymarin) (Katiyar et al 1997), the standard group (also containing equivalent dose) was treated with marketed Phytosome formulation of silymarin while the control group was given a topical saline treatment. This dose was decided after preliminary dose-response studies and through literature, which indicated that 900 μg dose was sufficient for protection from acute UVB-induced inflammatory responses in the skin.

5.4.2. Radiation exposure

The source of UV B used was a UV lamp (Hitech Ultraviolet Pvt. Ltd. Mumbai, India) placed 15 cm away from animal skin which emitted UV rays in the range of 290-320 nm with an output peak at 312nm. The energy output of the lamp was measured with a UV light meter (UV 340 A, Lutron Electronics). All animals were shaved on their back before radiation exposure. Animals were exposed to UV radiation two times a day for a week with a UV B dose of 0.115-0.23 J/cm\(^2\) (Learn et al 1995) (Mitchell et al 2011) (Mcglade et al 2007)

5.4.3. Evaluation of wrinkles formation

After seven days of UV exposure, the condition of animals was evaluated. After seven days exposure of UVB irradiation, animals were anesthetized for evaluation of dorsal skin wrinkle formation. Skin wrinkles were analysed according to the scale mentioned in literature (Anthony et al 1994) (Xia et al 2000) (Saupe et al 2005) (Stecova et al 2007). Wrinkles formation was observed in blind fashion for each animal, as described in Table 38.
Table 38: Grading of rat dorsal skin wrinkles according to the scale

<table>
<thead>
<tr>
<th>Score</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No wrinkles</td>
</tr>
<tr>
<td>2</td>
<td>A few shallow wrinkles across the back skin are observed occasionally</td>
</tr>
<tr>
<td>4</td>
<td>Shallow wrinkles across the back skin are observed on the whole surface</td>
</tr>
<tr>
<td>6</td>
<td>Some deep, long wrinkles across the back skin are observed</td>
</tr>
<tr>
<td>8</td>
<td>Deep, long wrinkles across the back skin are observed on the whole surface</td>
</tr>
</tbody>
</table>

5.4.4. Determination of skin fold thickness and histology

Dorsal skin edema induced by acute UVB exposure was calculated by skin fold thickness at harvest, using digital callipers. The inflammation was determined by the amount of skin edema at the exposure side of UV irradiation (Xia et al 2000) (Saupe et al 2005). For histological evaluation, skin samples were collected at the end of experiment and fixed with 10% v/v buffered formalin solution. Paraffin embedded section were stained with eosin and haematoxylin solution and examined using an optical microscope.

Based on the lower value of Inhibition concentration (IC₅₀ : 21µg/ml) and higher anti oxidant property of silymarin, in vivo activity of silymarin-NLC was evaluated at mice skin by western blot and RT-PCR study.

5.5. Anticancer study at enzyme level

5.5.1. Animals, diets and chemicals

Female Swiss albino mice were obtained from the in house animal breeding colony at CSIR-IITR Lucknow (IAEC approval IITR/IAEC/04/2015), India. Animals were quarantined for one week and fed with synthetic pellet diet (M/S Provimi Animal Nutrition India Pvt. Ltd.)
Bangalore) and water *ad libitum*. Animals used in the study were handled as per norms of Institutional Animal Ethics Committee. DMBA were procured from Sigma Co. St. Louis, MO,USA.

5.5.2. Method

Mice were divided into five groups, consisting of 12 animals each. Two days prior to study, interscapular region of back side of skin was shaved by using an electric clipper. All treatments were given topically on shaved area of animals. Treatment details are mentioned in Table 39, where placebo stands for plain NLC gel, Inositol Phosphate (IP₆) 5mg in acetone was used as standard drug, NLC 9 was the optimized Silymarin nano formulation. To compare effect of encapsulated silymarin in NLC and free silymarin, group 5 was studied. Three animals from each group were sacrificed at 4th, 8th, 16th and 24th hr subsequent to treatment. Skin was expurgated, snaps frozen in liquid nitrogen and stored at -80° for further experiments.

Table 39: Treatment details of optimised Silymarin NLC 9 gel

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Groups</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Group I (Negative control)</td>
<td>Placebo formulation</td>
</tr>
<tr>
<td>2</td>
<td>Group I (Positive control)</td>
<td>DMBA+ Placebo formulation</td>
</tr>
<tr>
<td>3</td>
<td>Group I (Standard)</td>
<td>DMBA+ IP₆ in acetone</td>
</tr>
<tr>
<td>4</td>
<td>Group I (Test)</td>
<td>DMBA+ NLC 9</td>
</tr>
<tr>
<td>5</td>
<td>Group I</td>
<td>DMBA+ Silymarin</td>
</tr>
</tbody>
</table>

5.5.3. Western Blot study

Epidermal tissue was extracted from mice, by freeze–thaw method and quantified by utilising Bradford protein assay technique. Tissue lysate of 10% was prepared in 20 mM Tris buffer of pH 7.5, having sucrose (250 mM), TrisCl (20mM), MgCl₂ (2mM), Ethylenediamine tetra acetic acid 2mM, Ethylene glycol tetraacetic acid (EGTA) 0.5 mM, Dithiothreitol (DTT) 100 mM, Na₃VO₄ (30 mM), Phenylmethysulfonyl fluoride (PMSF) 100 mM and protease...
inhibitor cocktail (Sigma). Tissue lysate equivalent to 50 μg proteins was resolved on sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) using 10% gels. Segregated proteins were shifted onto methanol soaked Polyvinylidenedifluoride (PVDF) membrane (Millipore Co.) and probed with ODC, Proliferating cell nuclear antigen (PCNA), Cyclin D1 and COX 2 primary antibodies followed the horseradish peroxidase conjugated secondary antibodies (Bangalore Genie). Antibody binding signals were envisaged by Chemiluminescence horseradish peroxidase (HRP) detection system (Millipore) on Versa Doc (Bio-Rad) and band strength was reckoned by Syngene gene tool. β-actin antibody (Sigma Co, USA) was used as loading control for every blot after stripping (Figure 34) (Tiwari and Gupta 2014) (Wolf et al 2000) (Wu et al 2012)

Figure 34: Protective effect of Silymarin in NLC 9 gel on DMBA induced deregulation of ODC, PCNA, COX-2 and Cyclin D1. Qualitative and quantitative analysis of ODC, PCNA and COX-2 and Cyclin D1 at protein level (n=3) is shown in (I) and at mRNA level in (II), I-Placebo formulation, II-DMBA+ Placebo formulation, III-DMBA+IP6 in acetone, IV-DMBA+NLC gel, V-DMBA+ Silymarin
5.5.4. Reverse transcription polymerase chain reaction (RT-PCR)

Samples were examined for their mRNA (messenger Ribonucleic acid) content by RT-PCR. Total RNA was extracted via Trizol reagent (invitrogen) in accordance with the supplier instructions and Deoxyribonucleic acid contaminations were seprated through DNaseI (Ambion Co) treatment. c-DNA was synthesized from total RNA by taking 2ng RNA equivalent amount by using preparation kit. Subsequent to c-DNA synthesis, quantification of particular mRNA in above mentioned genes was performed through mice specific primers, as specified in Table 40 (MWG Bio Tech, Germany), 20µl of reaction mixture restrained 100 ng of c-DNA, 1.5 mM of MgCl2, 10 pM each primer, 1.5 mM of dNTPs, and 1 unit AmpliTaq DNA polymerase enzyme (Bangalore Genie). Product was then augmented through thermal cycles (denaturation at 95 °C for 5 minutes, 95 °C for 60 seconds, annealing temperature for 60 seconds and at 72 °C for 60 seconds) × 35 subsequently final extension of 72 °C for 4 minutes through My-Cycler Thermal Cycler (Bio-Rad). PCR products thus obtained were resolved and visualized over 1.5% agarose gel containing ethidium bromide. Quantification was done via gene tool Syngene software (Table 40).

Table 40: Nucleotide sequences used for RNA analysis by RT-PCR for NLC 9 gel

<table>
<thead>
<tr>
<th>Sr. no.</th>
<th>Gene</th>
<th>Sequence</th>
<th>Product size (bp)</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PCNA</td>
<td>F 5′-GAAGCACCAAATCAAGAGAA-3′ R 5′-TCACCCCATATTCTTGACACAG-3′</td>
<td>193</td>
<td>55</td>
</tr>
<tr>
<td>2</td>
<td>Cyclin D1</td>
<td>F 5′-TGTTCGTGGCCTCTAAGATGAAG-3′ R 5′-AGGTTCACCTTGGAGCTTGTCAC-3′</td>
<td>136</td>
<td>60</td>
</tr>
<tr>
<td>3</td>
<td>COX-2</td>
<td>F 5′-GTGGAAAAACCTCGTCAGA-3′ R 5′-TGATGGTGGCTGTGTTTGTA-3′</td>
<td>256</td>
<td>60</td>
</tr>
<tr>
<td>4</td>
<td>ODC</td>
<td>F 5′-TGGAGTGAAATCATAGCTG-3′ R 5′-TTGCCCTCTTGGAAAACCCTTG-3′</td>
<td>410</td>
<td>58</td>
</tr>
<tr>
<td>5</td>
<td>β-Actin</td>
<td>F 5′-TGTTGATTGGTGTTGAATGGTGTCAG-3′ R 5′-TTTGATGTCACGACAGATTTCC-3′</td>
<td>514</td>
<td>60</td>
</tr>
</tbody>
</table>
5.6. Histology
The skin was collected at the end of experiment and stored in 10% phosphate buffered formalin solution. After fixation, samples were dehydrated and embedded in paraffin. 5μm microtome sections of the skin were then stained with hematoxylin and eosin. The light microscope (Olympus BX40) equipped with computer-controlled digital camera was used to visualize the images on the slides (Figure 35).

Figure 35: Effect of topically applied Silymarin-NLC 9 gel on UV-induced skin Edema in rat skin: UV induced skin without any treatment and after treatment of Silymarin NLC gel (from top to bottom)
5.6. Results and Discussion

5.6.1. Ex Vivo Studies

5.6.1.1. Skin permeation study comparison with marketed formulation

In vitro permeation study was performed to compare the permeation of silymarin with the various NLC gel formulations. NLC 9 (optimized formulation) and SILYMARIN® P (marketed formulation) contained the same amount of silymarin. The cumulative permeation profile of the different formulations revealed that the permeation of silymarin from the NLC gel formulation was greater, compared to the permeation of silymarin from the SILYMARIN® P formulation. In NLC 9, 74% of silymarin permeated within 24 hr, whereas 57% of silymarin permeated from SILYMARIN® P (Figure 36). Permeability parameters like JSS, Kp and enhancement ratio were significantly higher in NLC 9 formulation than SILYMARIN® P. The cumulative amount of the permeated drug at the end of 24 hr was 3241.11, and 2495.940 µg/cm², with a steady state flux (Jss) of 169.22 and 58.67 µg/cm²/hr and permeability coefficient (Kp) of 1.023 and 0.0543 cm/hr for NLC 9 and SILYMARIN® P respectively.

![Figure 36: Comparative silymarin permeation profile of NLC 9gel and SILYMARIN® P (marketed)](image-url)
5.6.1.2. Cytotoxicity of Silymarin-NLC on SK-MEL-2 cells

The cytotoxicity of silymarin-NLC was found dose-dependent for the SK-MEL-2 cell line. The viability of cancerous cells was, very high when treated with silymarin-NLC even at low concentrations (from 20-60 μg/ml) (Miroliaee et al 2011). To ensure the anti-proliferative and anti-inhibitory activity of silymarin-NLC, these non-toxic doses were therefore eliminated for further in vivo experiments. At the end of 72 hr, SRB assay was performed and inhibitory activity was measured based on the cell number (Figure 37). Silymarin-NLC 9 showed 63% inhibition of cell in comparison to Silymarin-Phytosome, which showed 48.14%. Inhibition activity of silymarin NLC gel was also in agreement with previous studies which proved the potential of silymarin natural flavonoid for protection against UV B induced tumour (Rai et al 2012) (Katiyar et al 2005) (Chatterjee et al 1999) (Svobodov et al 2007). NLC 9 gel, Phytosome (marketed), positive control showed significant activity in SK-MEL 2 cell line with IC 50 value of 21 μg/mL, 28 μg/mL and 8 μg/mL respectively. As per National Cancer Institute (NCI, USA), 30 μg/mL is the upper limit of IC50, that is considered promising for a crude extract (Kuete et al 2013) (Akindele et al 2015).
Figure 37: Anticancer activity of silymarin on SK-MEL-2. Graph showing the percentage proliferative inhibition values of NLC 9 gel, Phytosome (marketed) and control on cell line. All values are average of triplicates

5.6.1.3. Silymarin-NLC inhibits the migration and invasion of cells

Silymarin-NLC inhibits the motility of cancerous cells: Silymarin-NLC displayed an inhibitory effect on cell motility for SK-MEL-2 cells after 24 h and 48 h of co-incubation at higher concentration (80 μg/ml) as evidenced by the lesser spindle like morphology after treatment, as compared with the untreated control (Figure 38). The inhibitory effect was found to be concentration-dependent. This was most marked for the concentration range of 80 μg/ml to 100 μg/ml.

Figure 38: Effect of Silymarin-NLC 9 gel on migration and invasion without any treatment and after Silymarin-NLC treatment (from left to right) on SK-MEL-2 cells after 48-hr treatment
5.6.1.4. Silymarin-NLC inhibits the proliferation of SK-MEL-2 cells

The cell morphology was analysed after 24 hr treatment with silymarin-NLC 9 gel at a test concentration of 80µg/ml. There were considerable morphological changes in the tested cell lines with inhibition of cell proliferation and cell death after drug treatment. In order to check the inhibitory activity of silymarin-NLC 9 gel on SK-MEL-2 (human metastatic cell line derived from skin of thigh) cell line, the cells were plated and treated with different concentration of the drugs (10, 20, 40, 80 µg/ml). Morphology of cells for SK-MEL 2 cell lines is shown in Figure 39. It was noted that SK-MEL 2 cells grow in clusters and with predominately spindle morphology and light pigmentation before Silymarin NLC 9 gel treatment. After treatment, retardation in cell attachment and spreading was observed; which proved inhibition of cell growth. The presence of apoptotic bodies could also be seen in the NLC gel treated cells. We observed that encapsulation of Silymarin in to lipoidal carrier showed higher inhibition of cell growth on SK-MEL 2 cell as compared to untreated cells. It has been verified that HHPH preparation process of NLC protects silymarin from degradation without affecting anticancer activity and increase its effectiveness. The enhanced activity of silymarin NLC 9 gel may be related to its better permeation and stable formulation and synergistic effect of excipients.
Figure 39: Cell line SK-MEL-2 after Silymarin NLC 9 gel application and before silymarin application (from top to bottom)
5.6.1.5. Cytotoxicity of Silymarin-5-Fluorouracil NLC on SK-MEL-2 cells

Silymarin-5-FU NLC were prepared to overcome the side effects associated with higher dose of 5-Fluorouracil and size related limitations of nanoparticles in chemotherapy. A cytotoxicity study on cell line SK-MEL 2 followed by SRB assay clearly demonstrated the efficiency of these nanoparticles to induce apoptosis in SK-MEL 2 cells. Morphology of cells for SK-MEL 2 cell lines is shown in Figure 40.

Cytoprotective activity of silymarin is related to antioxidative, radical-scavenging effects and modulation of cell signalling pathways. Effect of silymarin against UV irradiation cause up regulation of tumor-suppressor genes p53. Usually, enhanced level of p53 leads to extended G1 phase and cyclin-dependent kinase inhibitor, which permits sufficient time for the cell to repair the damaged DNA. Therefore, silymarin cause G1 arrest, after UVB exposure and facilitate DNA repair mechanism. Silymarin inhibits UVB-induced expression of G1 phase cyclin and cyclin-dependent kinases in skin and delayed the G1 phase progression (Karimi et al 2011). Silymarin also caused, depletion of catalase activity and induction of cyclooxygenase and ornithine decarboxylase activity. This effect provides protection against photocarcinogenesis. 5-Fluorouracil causes inhibition of thymidylate synthase which is responsible for conversion of deoxyuridine to thymidine, resulting in reduced DNA synthesis, a decrease in cell proliferation, and the induction of cell death. The topical use of 5-Fluorouracil is a challenging in the treatment of skin cancers, because skin resists absorption, concentrations of 5-FU that can be used on the skin. Taking care that, very large quantities of active chemicals can be applied to skin in NLC form without any risk to the whole body. 5-FU destroys the malignant cell and spares the normal cell without systemic absorption (Dana et al 2009). It is more effective in antitumor activity since it has to inhibit tumor cells by competitive inhibition of thymidylate synthesase. Alternately, 5-FU may act by preferential incorporation into RNA to undergo inflammatory reactions, making fraudulent RNA. In photo damaged skin it triggers a cascade of fibrogenic activities of the dermis, benefit of a restorative effect from sun damage.
Figure 40: Cell line SK-MEL-2 before silymarin-5-fluorouracil gel application and after silymarin-5-fluorouracil gel application (from top to bottom)
At the end of 72 hr, SRB assay was performed and inhibitory activity was measured based on the cell number. Silymarin-NLC showed 67.53% inhibition of cell in comparison to Silymarin-5-Fluorouracil-NLC, which showed 72.04%. Silymarin-NLC gel, Silymarin-5-Fluorouracil-NLC gel and positive control showed significant activity in SK-MEL 2 cell line with IC₅₀ value of 20 μg/mL, 11 μg/mL and 9 μg/mL respectively. Silymarin-5-Fluorouracil-NLC gel (IC₅₀ 11 μg/ml) resulted in an increase in % inhibition, as compared to Silymarin-NLC gel. The significant difference (nearly 2 times) in the IC₅₀ values of combination of both natural and synthetic actives was obtained. This was because of mode of action of these actives in skin cell were entirely different. Based on the observation that the IC₅₀ values of these compounds were less than 30 μg/ml, both were considered very active for protection against Photoinduced tumourigenicity. The effects of Silymarin-NLC gel, Silymarin-5-Fluorouracil-NLC gel is concentration dependent. The results obtained in this study confirmed that 5-fluorouracil result in apoptotic cell death and effective against human melanoma SK-MEL-2 cells.

As we obtained IC₅₀ value (11 μg/mL) for silymarin-5-Fluorouracil-NLC, proved that they act as protectant and cause inhibition in progression. LC₅₀ is a measure of the lethal dose of a substance. The LC₅₀ endpoint calculates the concentration that kills 50% of the cells that were present at the time of drug addition (Zhang et al 2007). A lower LC₅₀ is indicative of increased cytotoxicity. LC₅₀ of positive control (48.6 μg/ml), whereas silymarin-5-Fluorouracil did not show any sign of activity. Based on this observation (LC₅₀ > 1000 μg/ml), we can conclude that silymarin-NLC and silymarin-5-Fluorouracil NLC, did not show any cytotoxicity to cancerous cell as similar to silymarin-NLC.

This data demonstrate that the lethal rate against cancerous cell of silymarin is not promising. Similarly, its combination with 5-Fluorouracil, cytotoxicity against cancerous cell was (>1000 μg/ml) also not in activity range. Morphologically no sign of dead cells were observed. So this combination NLC did not show any difference in LC₅₀, so both are devoid of cytotoxicity against cancerous cell, so can not be used for treatment of Photoinduced tumour and act as a protectant only against Photoinduced tumourigenicity.

Silymarin-5-Fluorouracil-NLC gel showed reduced activity when used in combination. Lethal activity was not enhanced significantly, in combination. *In vivo* studies were not conducted for Silymarin-5-Fluorouracil-NLC gel, but it is possible that entrapment of 5-
fluorouracil with silymarin may result in reduced toxicity and in turn reduced side effect profile.

5.6.2. *In vivo* studies

5.6.2.1. Skin Irritation Study

The skin irritation effect of the gel was graded A, which implies no reaction at 3, 5 and 7 days interval. The results of the skin irritation study showed that, by application of silymarin NLC 9 gel, there was no reaction on the skin.

After 7 days, on treatment with the Silymarin-NLC gel (500mg/rat), the score for skin irritation in terms of erythema and edema in all rats was found to be from 0 – 1 and the same result has been summarised in Table 41. On sites applied with NLC gel, erythema with scores of 2 and more than 2 were not found in any animals. In terms of edema, none of the animals indicated edema formation after the first application of any formulation. At the end of 7 days none of the animals showed either erythema or edema. At 5 days, one rat has very slight erythema but it healed at the 7th day. Evaluation of irritation of pharmaceutical topical products with natural actives is a significant step in the evaluation of their biocompatibility. Researchers and regulatory agencies recognize the important role of in vitro and animal tests play in the biologic evaluation of topical formulations. Rats and human skins have slightly different physiological properties and different responses to environmental and chemical agents. Despite the increasing evidence of differences of the skin responses between rats and human, data based on rat skin test have been taken as a reference to determine the irritant potential of formulation with an exception of rare published studies comparing results obtained both from animals and humans.
Table 41: Observation of erythema and edema after application of silymarin-NLC9 gel

<table>
<thead>
<tr>
<th>Animals</th>
<th>Reactions</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Erythema</td>
<td>3 days</td>
</tr>
<tr>
<td>Rat 1</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Rat 2</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Rat 3</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Edema</td>
<td>0</td>
</tr>
<tr>
<td>Rat 1</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Rat 2</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Rat 3</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

5.6.2.2. UV-induced wrinkles and thickening in rat skin

After 7 days of UV irradiation, the epidermal thickness of animals increased while the thickness decreased after silymarin-NLC application, in a dose dependent manner. This confirmed that silymarin-NLC 9 might possess an inhibitory effect on this kind of hyperplasia caused by UV irradiation. NLC 9 showed activity up to 24 hr., which could be due to the nano lipoidal system. Wrinkle scores were higher in control group of animals as compared to Silymarin NLC gel treated animals. In control group rats score of 6 was recorded (some deep long wrinkles) and 8 (for deep long wrinkles across the whole surface on back skin, while in Silymarin NLC gel and Phytosome (marketed) treated animals we observed score 2 (few shallow wrinkles), Statistically significant (P < 0.05) difference in the activity of NLC gel was observed, when compared with marketed formulation at the end of 24 hr. However, marketed formulation showed increased activity from 2 to 5 hr, and diminished activity at the end of 8 hr. The initial rapid onset of action of the silymarin NLC gel was proved by more than 50% inhibition at the end of 3 hr. and 70% inhibition at the end of 5 hr., which was better than % inhibition obtained from commercial formulation (Figure 41).
5.6.2.3. Histology evaluation

In the skin of rats several microscopic effects were observed. Microscopic images showed that UVB exposure induced acanthosis (thickening of stratum spinosum) hyperkeratosis, and an inflammatory response in rats. Regarding histopathological changes erythema, edema, redness, skin thickening and wrinkles proved that rats were sensitive to UVB exposure at applied radiation dose. However, these UV-exposed skin changes were partially restored by treatment with topical silymarin-NLC 9. (Soumya et al 2011) (Arias et al 2011)

5.6.2.4. Anticancer study at enzyme level

Silymarin prevents both photocarcinogenesis and skin tumor promotion by antioxidant action. (Mussi et al 2013) It inhibits mitogenic and cell survival signalling and induces apoptosis. (Singh et al 2014) (Spada et al 2013) (Toklu et al 2007). It is well established in literature, that inflammation is linked to carcinogenesis and acts as a driving force in premalignant and malignant transformation of cells (Lai et al 2008). It has been observed that cyclooxygenase-2 (COX-2) is upregulated after UV exposure to skin cells and is involved in the development of skin cancer (Abel et al 2009) (Pan et al 2012) (Tripp et al 2003) Thus, the
interruption of COX-2 is an effective strategy to prevent and inhibit skin cancer. Topical application of DMBA to mice leads to edema and papilloma formation by enhancing COX-2 and ornithine decarboxylase (ODC) protein expression (32-34). In this study, we investigated DMBA stimulated upregulation of COX-2 and ODC messenger ribonucleic acid (mRNA) and protein expression in mice skin and its chemoprevention by topical application of Silymarin NLC.

5.6.2.4.1. Inhibitory effects of Silymarin-NLC 9 gel on DMBA-induced, ODC and PCNA expression in mice skin

ODC protein is a rate-limiting enzyme in the synthesis of polyamines that play a major role in cell growth and proliferation (Tang at al 2004) (Elmets et al 2010). Restriction of ODC enzyme activity, inhibits growth of skin tumors. ODC regulation was found altered after DMBA application. Silymarin-NLC modify the process of cellular signalling and thus inhibit initiation and progression of tumour, by its action on ODC. PCNA protein encoded by this gene is found in the nucleus and increases the process of leading strand synthesis during DNA replication (Dornelas et al 2009) (Jantschitsch et al 2009). The anti-inflammatory activity of NLC can be demonstrated by its effect on down regulation of ODC and PCNA activity in DMBA-stimulated mice skin.

To study the effect, quantitative analysis of ODC and PCNA at mRNA level was observed. To determine the effect of NLC 9 gel on cellular proliferation/progression, ODC activity was measured at 4hr, 8hr, 16hr and 24hr time points (Figure 42). Assuming negative control group levels to be baseline, ODC level was raised by DMBA induction to 67% while in presence of NLC it was found to be 48%. PCNA was down regulated to 49% from 52% for negative control.

5.6.2.4.2. Inhibitory effects of Silymarin-NLC 9 gel on DMBA-induced, COX-2 and Cyclin D1 expression in mice skin

COX-2 expression was evaluated by Western blot and RT-PCR. These analyses showed that after silymarin-NLC treatment, the expression of the COX-2 protein was substantially decreased in DMBA-induced mice skin (Figure 43). Cyclooxygenase-2 (COX-2) is induced by inflammatory cytokines, growth factors, and tumor promoter’s agents, and is upregulated in a
skin malignancy and supports the growth of malignant cells by stimulating proliferation. These results proved that COX-2 plays an important role in the generation and progression of tumors, and inhibition of COX-2 may stop the growth of a variety of tumours. In addition, many research have proved that COX-2 inhibition therapy could be beneficial in the prevention of skin cancer. One of the main anticancer mechanisms of silymarin is to decrease production of inflammation biomarkers in the tumor, resulting in an inhibition of proliferation and induction of apoptosis in tumor. Cyclin D1, related with enhanced proliferation, is one of the cell cycle proteins responsible for transition to the S phase of the cell cycle, and its overexpression is related with malignant transformation. Various research also showed that cyclin D1 plays a significant role in regulating cell cycle progression in skin cancer cells and that destruction of cyclin D1 is sufficient to induce G1 cell cycle arrest. The expression of cyclin D1 responsible for progression of tumorigenesis of human skin. Therefore, it has been assumed that a decrease of cyclin D1 could be effective in inhibition of proliferation of tumor cells.

In this research we evaluated the expression of cyclin D1 in DMBA-induced mice and the relationship between the antitumor effect of silymarin-NLC and the expression of cyclin D1. We investigated this relationship because research had proved that COX-2 and cyclin D1 were both up-regulated in Photoinduced tumour.

As illustrated (Figure 42 & 43), topical application of NLC 9 gel to DMBA induced mice skin, resulted in down regulation in the levels of COX-2, and Cyclin-D1 proteins. A statistically significant (P < 0.001) suppression of COX-2 and Cyclin-D1 gene expression in mice skin was noticed through western plot and PCR analysis. As shown in Figure 42, silymarin-NLC significantly decreased the mRNA expression levels of cyclin D1 compared with the control (P < 0.05), and it also decreased the expression of COX-2 at mRNA levels. DMBA induced mice showed upregulation of COX 2 up to 69%, however in presence of standard drug and NLC the upregulated level was found to be 49 % at the end of 24 hour. In PCR analysis, it was observed that in presence of DMBA, COX 2 increased up to 67%, while in presence of NLC it was found to be 43%. DMBA induced mice showed 69% upregulation of Cyclin D1, while in case of NLC it was found to be 63%. NLC showed 6% control on upregulation of Cyclin D1. It can thus be concluded that NLC regulated the Cyclin D1 level to a lesser extent as compared to COX 2 level. Cyclin D1 upregulation was less controlled by NLC as was confirmed by PCR study also. Moreover, according to various reports DMBA/TPA exposure for short term, edema or hyperplasia increased with time up to
12-16 hour then starts decreasing. Due to this, a fall was observed in expression of COX-2, PCNA and ODC (Cipolat et al 2014) (Hara et al 2005)

Topical application of DMBA to mice leads to edema and inflammatory effect by enhancing COX-2 and ODC protein expression. It was demonstrated that topical application of Silymarin NLC 9 gel effectively inhibited DMBA-stimulated transcription of inducible COX-2 and ODC mRNA and protein expression in mice skin. In the current research we clearly demonstrated that silymarin-NLC application downregulates the enhanced level of ODC, PCNA, COX 2, Cyclin-D1 enzyme. Targeted inhibition of inflammation markers was considered a promising approach to stop formation of tumour. Inhibitory action of silymarin-NLC in mice skin suggests that starting effect may be the inhibition of inflammatory response which may result in inhibition of tumour promotion. Uncontrolled cell proliferation is necessary for Photoinduced tumour growth and progression. Cancer cells have an advantage due to deregulation of cell cycle proteins and enzymes, causing abnormal growth that lead to tumor development. In this study, we have shown that the tumor growth inhibition by silymarin-NLC was accompanied by a fall in proliferation rate, and that low dose was insufficient to control while high-dose of silymarin showed greater effect in proliferation inhibition of photo induced tumor.
Figure 42: Inhibitory effect of Silymarin NLC 9 gel on DMBA-induced activity in albino mice, COX-2, Cyclin D1, ODC and PCNA activity at protein level was measured by western blot analysis. Each data bar represents the mean ± SD of four mice; each sample was assayed in triplicate. I- Placebo formulation, II- DMBA+ Placebo formulation, III- DMBA+ IP6 in acetone, IV- DMBA+NLC, V- DMBA+Silymarin. Bars represent the standard deviation. * ‘p’ < 0.05, ** ‘p’ < 0.01, *** ‘p’ < 0.001 with respect to positive control. # ‘p’ <0.05, ## ‘p’ <0.01, ### ‘p’ < 0.001 with respect to DMBA, when the comparison was done between the effects of individual with that of combination treatment at different time points.
II

**CYCLIN D1**

![CYCLIN D1 Graph](image)

III

**ODC**

![ODC Graph](image)
Figure 43: Inhibitory effect of Silymarin NLC 9 gel on DMBA-induced activity in albino mice, COX-2, Cyclin D1, ODC and PCNA activity at m-RNA was measured by PCR analysis. Each data bar represents the mean ± SD of four mice; each sample was assayed in triplicate. I- Placebo formulation, II- DMBA+ Placebo formulation, III- DMBA+IP6 in acetone, IV- DMBA+NLC-V-DMBA+Silymarin. Bars represent the standard deviation. * ‘p’ < 0.05, ** ‘p’ < 0.01, ***‘p’ < 0.001, with respect to positive control. # ‘p’ < 0.05, ## ‘p’ < 0.01, ### ‘p’ < 0.001 with respect to DMBA, when the comparison was done between the effects of individual with that of combination treatment at different time points.

The presence of skin barrier is one of the challenges for drug development of skin cancer. Silymarin-NLC was used to enhance permeability across skin layer and to provide targeted delivery to inflammation biomarkers. Research studies with enhanced permeability and in vitro inhibition of proliferation was proved in these studies by sulfo rhodamine-B (SRB) assay. To gain further insight and to further check activity of silymarin-NLC as a topical delivery using lipoidal approach was done by genetic study. Treatment with DMBA in mice is widely used model for skin cancer study. It was well proved that inflammation is linked to carcinogenesis and acts as a driving force in premalignant and malignant transformation of cells. This research was short term study, so single dose of carcinogen 7,12-dimethylbenz[a]anthracene (DMBA) was applied topically and there was no repeating doseof
DMBA. Genetic level changed were observed and shown by western blot and PCR images (Tiwari et al 2014) (Abel et al 2009). A short-term study for 24 h cannot be used to show treatment effect for cancer therapy but many evidences showed that different carcinogens in higher amount can upregulate the levels of various proteins such as ODC, COX-2 etc. These proteins were markers pertaining to cellular proliferation/ differentiation and inflammation, which ultimately lead to cancer (Gupta 2012) (Gupta and Rani 2001). It was stated that any agent reducing their upregulation can reduce or subside cancer initiation, as found in present study. These results can be further examined by conducting a long term anticancer study. In order to evaluate the molecular basis of anti-proliferative effects of NLC 9 the deregulation of ODC and PCNA was considered at protein and mRNA level before and after DMBA treatment. Assessment of impact on proliferation and cell cycle regulators was also attempted. Alterations in treated groups were compared with that of untreated control.
References


