IN VITRO DRUG RELEASE CHARACTERISTIC AND CYTOTOXIC ACTIVITY OF SILIBININ-LOADED NOVEL GEL FORMULATION

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ABSTRACT

Introduction: Silibinin is a potent antioxidant and anticancer activity which protects skin against UV-B rays. The objective of the present work was to formulate a novel stable gel formulation which helps to increase the therapeutic value by better permeation, anticancer action and reduced toxicity. Material and method: The drug and complex were characterized by phase solubility study, drug content, solubility studied solid-state characterization by DSC, FT-IR, dissolution studies and in vitro permeation studies and in vitro activity of formulation was carried out by MTT Assay respectively. Result & Discussion: The all physicochemical test for complex (1:1 ratio) and gel was performed which were meet required limits. In vitro cytotoxicity study of drug in gel and complex in gel tested against HaCaT cell line showed marketed cytotoxicity having IC50 value 165.1 & 158.7 µg/ml respectively. Conclusion: The topical stable herbal gel formulation shows anticancer activity against the skin Keratinocyte cell line.

Keywords: Silibinin, Gel, cytotoxicity, skin cancer

I. INTRODUCTION

In recent years, silibinin (SB) has received a great amount of attention as an herbal remedy to treat cancer and liver-related diseases. And also applicable in the treatment of liver disease, neurodegenerative, diabetes mellitus, asthma, gastrointestinal problems, nephropathy, skin disease alcoholic liver cirrhosis(1)(2). SB as the main component of silymarin is obtained from the Silybum marianum (milk thistle) belonging to family Asteraceae and has been used for a long time to treat liver disorders due to its potent hepatoprotective effect (4). However, it's low solubility in an aqueous environment which leads to poor bioavailability in the human body has limited its clinical potential in biomedical applications(5)(6)

The UV light that reaches the earth’s surface due to depletion of the ozone layer Both UVA and UVB cause wavelength-dependent damage to human skin including skin cancer, whose incidence is dramatically increasing(7). Recent studies showed that silymarin constituents could strongly protect against photo photo carcinogenesis inhibit UVB and chemical tumor promoter-induced skin inflammation and edema (7)(9)(10)(11) Silymarin also attenuates UVA-induced damage to human keratinocytes(12)(13)
II. MECHANISM OF ACTION OF SILYMARIN

Flavonoids act by preventing the interaction between chemical carcinogens and DNA(14)(15). Flavonoids were also reported to directly inhibit the prooxidant enzymes, lipoxygenases (LOX), COXs, and xanthine oxidase (XO). In brief the UV rays from the sun is strongly absorbed by cellular DNA in skin which damage DNA. The Flavonoids which having highly antioxidant property then acting by oxidative stress .which generate free radicals and reactive oxygen species (ROS). (14) (17)(4) these Flavonoids removing the moieties and protect against UV rays.(16) (13) (4)

In cancer, Chemotherapy is the drug treatment using powerful chemicals, and it is expected that kill the cancer cells for maximum treatment efficacy without destroying normal cells in the body. However, many of the conventional chemotherapeutic agents were associated with side effects like fatigue, nerve damage, nausea, hair loss, skin and nail changes, heart trouble, and sometime which can lead to serious complications (18). Due to this problem herbal due to having attention in cancer treatment. Silymarin decrease lipid peroxidation, decrease DNA synthesis in tumor cell (19), and exhibit prevention effect against photo-carcinogenesis in skin cancer. (5)(20) (18) Silymarin also administration problems like lack of selectivity, limited solubility, poor distribution, low oral bioavailability(21)(22). Now requirement to overcome all these problems during formulation so, In this we increase solubility and also give protection to the drug.

Gels are the semisolid system which gives a direct application of drug on site of action and gives localized treatment in skin disease these formulation having some advantages I.e. (23)

- Avoids gastrointestinal (GI) drug absorption difficulties caused by GI pH, enzymatic activity and drug interactions with food, drink, and other orally Administered drugs.
- Avoids the first-pass effect, possibly avoiding the deactivation by digestive and liver enzymes.
- Reduction of doses as compared to oral dosage forms.
- Ability to dissolve a wide range of medications with different chemical properties, making combination therapy with one transdermal cream possible.
- Provides extended therapy with a single application, improving compliance.
- Drug therapy may be terminated rapidly by the removal of the application from the skin surface.
- Less greasy and can be easily removed from the skin.(24) (25)

Disadvantages

These include the following

- Skin irritation due to drugs or excipients
- Poor permeability through the skin for some medications
- This route is not suitable for drugs with a large particle size
The possibility of allergic reactions
The denaturation of drugs due to the enzymes found in the epidermis

Material and method

The drug and other excipients are purchased from different suppliers which were listed below table no. 1 (list of chemicals)

Instruments

The instrument which was listed in following table was used in experiment Table no. 2 (List of instrument)

Method

Phase solubility analysis for silibinin

Phase solubility studies were performed to determine the stoichiometric ratio silibinin to β-cyclodextrin for this

- A stock solution of β-cyclodextrin was prepared using Distilled water from this stock solution, 1-6 M solution was prepared
- 5 ml from each molar solution was transferred in a volumetric flask and in it add an excess quantity of drug separately
- The volumetric flask was shaken at an ambient temperature, for 48 hours, using a laboratory shaker
- Then the solution was filtered carefully, the concentration of a solution was determined with UV spectrophotometer at 287 nm

III. PREPARATION OF SILIBININ GEL

1) Preparation of complex

To increase the solubility of silibinin and keep silibinin stable it necessary to formulate its complex (26)(2). According to the phase solubility study, the 1:1 drug and beta-cyclodextrin complex was prepared by a solvent evaporation method (27)(28)

2) Characterization of complex

a) Drug content estimation

The weight of inclusion complex equivalent to 100 mg of silymarin was dissolved in an appropriate solvent and measure the absorbance by UV spectrophotometer by calibration curve method at wavelength 288 nm. (29) (30)

The various method for preparation inclusion complex is compared

b) Dissolution study of silymarin and complex

Dissolution study of silymarin and inclusion complex was studied using USP-n dissolution apparatus (Electrolab). The dissolution was carried out in 900 ml of pH 7 phosphate buffer at speed 75 rpm (33). Aliquots of 5ml were withdrawn periodically and this media was replaced with fresh 5ml phosphate buffer. (31)(32). The concentration of drug was determined by the calibration curve method at 288 nm

c) FTIR analysis

Fourier transform infrared (FTIR) measurements were performed on a shimadzu instrument (model FTIR-8400S)(27). The FTIR spectra of the samples were recorded in the scanning range of 400–4000 cm−1 with 42 scans at a resolution of 2 cm−1 using attenuated total reflectance technique (5) (29)

d) DSC

Differential scanning calorimetry (DSC) were analyzed thermal behaviors of the samples using a differential scanning calorimeter. Samples (5–10 mg) were sealed in a specialized aluminum pan using an aluminum lid with a pinhole (34). Measurements were performed over 0°C–230°C under a dry nitrogen atmosphere at a heating rate of 10°C/min.(20)(5)
3) Preparation of gel

The 1% w/w silibinin was taken for preparation of gel in which methanol is used as co-solvent. Weigh an accurate amount of carbopol and dispersed an insufficient amount of water. (35) Glycerin is added to it. And adjust the pH by addition of triethanolamine between the 6.8 to 7.2, then required amount of silibinin was dissolved in a small quantity of methanol. Then propylparaben is added to the formulation. Finally, Tween 80 was added to it.

4) Characterization of gel

a) Measurement of pH

The pH of various gel formulations was determined by using a digital pH meter. One gram of gel was dissolved in 100 ml distilled water and stored for two hours. The measurement of pH of each formulation was done in triplicate and average values are calculated. (28)

b) Drug content

1 g of the prepared gel was the mix in 100ml of suitable solvent. Aliquots of different concentration were prepared by suitable dilutions after filtering the stock solution and absorbance was measured. Drug content was calculated using the calibration curve method. (36)

c) Viscosity study

The measurement of viscosity of the prepared gel was done with a Brookfield Viscometer (model DV-n +pro). The gels were rotated at 0.3, 0.6 and 1.5 rotations per minute. At each speed, the corresponding dial reading was noted (28). The viscosity of the gel was obtained by multiplication of the dial reading with factor given in the Brookfield Viscometer catalogs.

d) Spreadability

It indicates the extent of the area to which gel readily spreads on application to the skin or affected part. The therapeutic potency of a formulation also depends upon its spreading value. (37) About 1 gm of gel formulation was weighed and kept at the center of the glass plate of standard dimensions (10x10cm) and another glass plate placed over it carefully, that the gel was sandwiched between the two slides. 2 kg weight was placed at the center of the plate (avoid sliding of the plate). The diameter of the gel in cms, after 30 minutes was measured and the results were tabulated (28)

e) Extrudability study

After the gels were set in the container, the formulations were filled in the collapsible tubes. The Extrudability of the formulation was determined in terms of weight in grams required to extrude a 0.5 cm. ribbon of gel in 10 seconds. (28)

f) In vitro Diffusion studies

The diffusion studies of the prepared gels can be carried out in Franz diffusion cell for studying the dissolution release of gels through a cellophane membrane (38) (36). Gel sample (0.5g) was taken in cellophane membrane and the diffusion studies were carried out at 37 ± 1° using 250 ml of phosphate buffer (pH 7.4) as the dissolution medium. 5ml of each sample was withdrawn periodically at 15, 30, 45, 60, 75, 90, 105, 120, 135 min. and each sample was replaced with an equal volume of fresh dissolution medium. Then the samples were analyzed for the drug content by using phosphate buffer as blank. (39)(40)(41).

g) Stability

The stability studies were carried out for all the gel formulation on Remi equipment here, by subjecting the product to a temperature of 4°C for 1 month, then at 25°C for 1 month and then at 37°C for 1 month, syneresis was observed. (9)(10). After this, the gel is exposed to ambient room temperature and liquid exudates separating is noted (33).
h) Photostability study

The photostability of silibinin gel and a methanolic solution of silibinin were compared using a reported method. Briefly, 10 ml of silibinin gel solution and methanolic solution (concentration 2-10 µg/ml) were exposed to UV light and noted the absorbance spectra of each sample just after the sample preparation as well as at the time interval of 5, 30, 60, 90, 120, 180 min respectively. At 288 nm in triplicate (42)(3).

5) MTT cytotoxicity assay

The cytotoxicity studies for HaCaT (normal human Keratinocyte) cell line carried out at Skanda life science Pvt. Ltd, DSIR recognized R & D center, Bangalore (32) (5).

IV. RESULTS AND DISCUSSIONS

Phase solubility analysis of silibinin

Phase solubility diagram (fig no. 1) of complex indicating an increase of solubility for silibinin with the increase in the beta-cyclodextrin concentration shown in table no.1. The shape of phase solubility curve may indicate that a 1:1 molar ratio is most probable for formation of complex.

Table no. 1- Phase solubility analysis of silibinin

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Conc. of β-cyclodextrin(Mm)</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>0.461</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>0.498</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>0.532</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>0.578</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>0.612</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>0.678</td>
</tr>
</tbody>
</table>

![Fig 1. Phase solubility diagram](image)

Characterization of complex

a) Estimation of drug content in the complex -Inclusion complex prepared by solvent evaporation method showed 99.74% drug content(37). The various method was followed for preparation complex (36) but solvent evaporation method shows maximum drug content which shown in (Table no. 2)
Table no. 2- % Drug Content

<table>
<thead>
<tr>
<th>Sr No.</th>
<th>Preparation method</th>
<th>Drug in mg</th>
<th>% Drug content</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Physical mixing</td>
<td>8.121</td>
<td>81.21</td>
</tr>
<tr>
<td>2</td>
<td>Kneading</td>
<td>7.654</td>
<td>76.54</td>
</tr>
<tr>
<td>3</td>
<td>Solvent evaporation</td>
<td>9.974</td>
<td>99.74</td>
</tr>
</tbody>
</table>

b) Dissolution study - The inclusion complex of silymarin with β-cyclodextrin produce enhancement in dissolution as shown in figure (fig no.2) (33)(43)

![Dissolution Curve: % Released vs. Time]

Fig 2. Dissolution Study

c) FTIR spectra- The IR spectrum of silibinin exhibited characteristic peaks in the table. The IR spectrum of silibinin–beta cyclodextrin complex demonstrated characteristic peaks in the following table (Table no.3&4) respectively.

Table No.3- FTIR spectrum for Drug Silibinin

<table>
<thead>
<tr>
<th>Sr.No.</th>
<th>Peaks</th>
<th>Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2933 cm⁻¹</td>
<td>CH alkane stretch</td>
</tr>
<tr>
<td>2</td>
<td>1606 cm⁻¹</td>
<td>CO-Stretch</td>
</tr>
<tr>
<td>3</td>
<td>3412 cm⁻¹</td>
<td>OH Stretch</td>
</tr>
<tr>
<td>4</td>
<td>3020 cm⁻¹</td>
<td>CH aromatic stretch</td>
</tr>
</tbody>
</table>

Table No.4- - FTIR spectrum for silibinin–beta cyclodextrin complex

<table>
<thead>
<tr>
<th>Sr.No.</th>
<th>Peaks</th>
<th>Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2897 cm⁻¹</td>
<td>CH alkane stretch</td>
</tr>
<tr>
<td>2</td>
<td>3304 cm⁻¹</td>
<td>OH stretch</td>
</tr>
<tr>
<td>3</td>
<td>1633 cm⁻¹</td>
<td>CO stretch</td>
</tr>
<tr>
<td>4</td>
<td>1460 cm⁻¹</td>
<td>C=C stretch</td>
</tr>
<tr>
<td>5</td>
<td>2860 cm⁻¹</td>
<td>Alkenyl CH stretch</td>
</tr>
</tbody>
</table>

d) DSC Study - The DSC technique is generally used to detect the glass transition temperature (Tg), which depends on the molecular weight, entanglement, and chain-end composition of polymers(9). The DSC data confirmed that silibinin (Fig.3) showed a sharp endothermic peak at around 189.01°C, which is in close agreement with the previously reported value (14). Furthermore, the previously reported beta cyclodextrin DSC temp. was near about 90°C, but here in spectra the wide peak at 54.77° it shows that inclusion complex
V. CHARACTERIZATION OF GEL

1. Physic-chemical evolution-All physic-chemical parameter are meeting required specification which enlisted in the table no.5.(28)(37)

Table No.5 – Physiochemical Evaluation

<table>
<thead>
<tr>
<th>PH measurement</th>
<th>Batch no.</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A1</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td>A2</td>
<td>7.1</td>
</tr>
<tr>
<td></td>
<td>A3</td>
<td>7.2</td>
</tr>
<tr>
<td>Spreadability</td>
<td>A1</td>
<td>7.5 cm</td>
</tr>
<tr>
<td>Extrudability</td>
<td>A1</td>
<td>+++</td>
</tr>
<tr>
<td>Viscosity</td>
<td>A1</td>
<td>2396</td>
</tr>
</tbody>
</table>

2. in vitro diffusion-The diffusion study of complex incorporated in gel show 86.2% drug diffusion through the membrane which is give stabilization and protective effect to the drug and better % diffusion in membrane.(45)which listed in following table no. 6.

Table No.6–Diffusion study

<table>
<thead>
<tr>
<th>Sr.No</th>
<th>Time (min)</th>
<th>Absorbance (at 288nm)</th>
<th>Concentration (μg/ml)</th>
<th>Percentage drug release</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15</td>
<td>0.031</td>
<td>0.007</td>
<td>0.047</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>0.094</td>
<td>1.16</td>
<td>11.6</td>
</tr>
<tr>
<td>3</td>
<td>45</td>
<td>0.158</td>
<td>2.351</td>
<td>23.51</td>
</tr>
<tr>
<td>4</td>
<td>60</td>
<td>0.194</td>
<td>3.018</td>
<td>30.18</td>
</tr>
<tr>
<td>5</td>
<td>75</td>
<td>0.239</td>
<td>3.85</td>
<td>38.5</td>
</tr>
<tr>
<td>6</td>
<td>90</td>
<td>0.312</td>
<td>5.20</td>
<td>52.0</td>
</tr>
<tr>
<td>7</td>
<td>105</td>
<td>0.380</td>
<td>6.46</td>
<td>64.6</td>
</tr>
<tr>
<td>8</td>
<td>120</td>
<td>0.417</td>
<td>7.14</td>
<td>71.4</td>
</tr>
<tr>
<td>9</td>
<td>135</td>
<td>0.468</td>
<td>8.09</td>
<td>80.9</td>
</tr>
<tr>
<td>10</td>
<td>150</td>
<td>0.497</td>
<td>8.62</td>
<td>86.2</td>
</tr>
<tr>
<td>11</td>
<td>165</td>
<td>0.302</td>
<td>5.01</td>
<td>50.1</td>
</tr>
<tr>
<td>12</td>
<td>180</td>
<td>0.087</td>
<td>1.03</td>
<td>10.3</td>
</tr>
</tbody>
</table>

3. Stability -The 1 month stability testing study was carried out at 3 different condition which show stability of gel formulation and these formulation not changes parameter of as shown in tableno.7.(28) (46)
Table No7: Stability study physical evaluations parameter

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Room temperature</th>
<th>37±5°C</th>
<th>4-5°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visual appearance</td>
<td>Transparent</td>
<td>Transparent</td>
<td>Transparent</td>
</tr>
<tr>
<td>Initial</td>
<td>Transparent</td>
<td>Transparent</td>
<td>Transparent</td>
</tr>
<tr>
<td>Final</td>
<td>Transparent</td>
<td>Transparent</td>
<td>Transparent</td>
</tr>
<tr>
<td>pH</td>
<td>Initial</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Final</td>
<td>7.2</td>
<td>7.1</td>
</tr>
<tr>
<td>Viscosity (cps)</td>
<td>Initial</td>
<td>43,450</td>
<td>43,450</td>
</tr>
<tr>
<td></td>
<td>Final</td>
<td>43,450</td>
<td>43,450</td>
</tr>
<tr>
<td>Phase separation</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
</tr>
<tr>
<td>Leakage</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
</tr>
<tr>
<td>Drug content</td>
<td>0 month</td>
<td>104.72%</td>
<td>104.72%</td>
</tr>
<tr>
<td></td>
<td>1 month</td>
<td>104.77%</td>
<td>99.12%</td>
</tr>
</tbody>
</table>

4. **Photo-stability** The light exposure caused a sharp degradation of silibinin which directly incorporated in gel shows 36.45% degradation while the drug complex incorporated in gel shows 3.05% degradation in few min. The conclusion from this photo-stability study that the better photo-protection of silibinin in complex was effective that the drug without complex. This result has been shown in the figure (fig no.4)

5. **MTT assay** - (39) The cell morphology was analyzed after 24 hr. of treatment of gel at a test concentration of 10-320 µg/ml; there was inhibition of growth of the cancer cell with IC50 value 158µg/ml which shows in figure (fig no. 5)
Discussion:

Gel formulation was selected as the optimized formulation was more effective which is suitable for topical application and having good viscosity, Spreadability. The stability study shows that at room temperature and low temp. Also it stable. The diffusion of drug through the membrane in 2.30 hr was found 86.2% .the gel shows improved photo stability of silibinin as well as may shows UV-ray blocking effect on the skin. This photo stability effect was considerable due to silibinin was encapsulated in beta-cyclodextrin. Silibinin complex in gel shows better inhibition of cell proliferation in compare to silibinin directed incorporated in the gel

VI. CONCLUSION:

Previously the anticancer activity of silibinin was reported in the literature. In this research work, the main objective is to increase solubility and dissolution profile of silibinin and it is achieved. The topical formulation shows anticancer activity against the skin Keratinocyte cell line by in-vitro cytotoxicity activity in future the animal study may carry to gel exact result for anticancer activity then it may use commercially.

REFERENCE


Fig 5: MTT Assay using HaCaT cells

<table>
<thead>
<tr>
<th>Conc.µg/ml (Log X)</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^-50</td>
<td>2.210</td>
<td>2.200</td>
</tr>
<tr>
<td>10^-50</td>
<td>1.651</td>
<td>1.587</td>
</tr>
</tbody>
</table>


