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Research

Design and Characterization of Phytosomal Gel by Using *Cardiospermum Halicacabum* L. Leaf Extract for it's Anticancer Activity

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Abstract

This study focuses on the design and characterization of a phytosomal gel utilizing the aqueous soluble extract of *Cardiospermum halicacabum* L. leaves for its potential anticancer activity against Melanoma. Given the hydrophilic nature of the extract, phytosomes were employed to encapsulate it, utilizing the Rotary evaporation technique and factorial design for formulation. The optimized phytosomal batch exhibited a particle size of 51.7 nm and a zeta potential of -57.1 mV, as confirmed by Transmission Electron Microscopy, showcasing smooth and spherical morphology. *In vitro* skin permeation studies, conducted through fluorescence microscopy, demonstrated enhanced penetration into deeper layers of the skin. The *Cardiospermum halicacabum* phytosomal gel displayed significantly heightened cytotoxicity *in vitro* ($p<0.01$), with an IC50 value of 23.89 µg/mL, in contrast to the *Cardiospermum halicacabum* L. leaf extract (31.69 µg/mL). The refined phytosomal gel underwent a comprehensive evaluation, encompassing *in vitro* skin penetration, cytotoxicity, apoptosis, and live-dead cell assays, all of which underscored its substantial anticancer efficacy.

Keywords: *Cardiospermum halicacabum* L., Phytosomes, Skin cancer.

INTRODUCTION

There are many different forms of cancer that are widespread worldwide, according to the National Cancer Institute (NCI) in the United States of America. One out of every three individuals born in affluent countries have a diagnosed form of cancer, and this is affecting nearly 1.2 crore people around the world.[1] The type of cell that a cancer begins in can be used to classify different cancers. a) Carcinomas, b) sarcomas, c) leukaemia, and d) lymphomas are the four primary categories. Additionally, malignancies can be divided into two

groups based on where they first appear in the body: (A) primary cancers and (B) metastatic tumours. The growth of cancer in the skin has increased rapidly over the last few years.5 In people, it accounts for around 30% of all newly diagnosed cancer cases each year.[2] *Cardiospermum halicacabum* L. belongs to the Sapindaceae family Tropical and subtropical regions are rich in this herbaceous plant throughout the world. Due to the presence of several chemical components, the extract from this plant has demonstrated a variety of medicinal properties, including antibacterial, antifungal, antiparasitic, antidiarrheal, anxiolytic, rubefacient, antipyretic, anti-inflammatory, anticonvulsant, and anticancer. Ethanol, Chloroform and Methanolic extract of *Cardiospermum halicacabum* L.[3] are effective against cancer like melanoma, breast cancer, lung cancer and carcinoma. The majority of the active ingredients found in herbal medicines include flavonoids, glycosides, etc. Because of their larger molecular size, which prevents them from being absorbed through inactive diffusion, and as they are low lipid soluble, which stops them from crossing the lipid-rich outer membranes of enterocytes, drugs have a poor bioavailability. A novel drug delivery system in herbal medicine offers a fresh method in delivering traditional pharmaceuticals at the proper concentration, proper location, for appropriate length of time, as well as providing scientific proof to support the standardization of herbal drugs. Benefits and bioavailability of phytosomes, which are herbal formulations that absorb more readily than conventional phyto-molecules or botanical extracts, are higher. Notwithstanding having strong bioactivity *in vitro* and *in vivo*, a number of plant extracts and phytoconstituents have poor lipid solubility, the wrong molecular size, or both. As a result, their absorption and bioavailability in humans are insufficient. As a result, considerable work has gone into creating "phytosomes," a cutting-edge innovation in herbal delivery that is more effective than conventional extracts of herbs in terms of absorption and utilization. Phytosomes, a combination of natural active components and phospholipid(s), enhance the uptake of herbal extracts or isolated active compounds whether applied externally or taken internally.[4]

MATERIAL AND METHOD

The plant were collected Vital herbs, Delhi, Phospholipid from VAV life science Pvt. Ltd. Ratnagiri, Carbopol 934 from Research lab fine chem. Industries, Methyl paraben from SD Fine Chem Ltd. Mumbai

Experimental work

Compatibility studies using FTIR spectroscopy[5]

The use of FT-IR allows researchers to examine and foresee any physico-chemical interactions or incompatibilities between various components inside a formulation via comparison of the IR spectra peaks of pure extract and the physical mixing of the extract and other excipients. As a result, it can be utilized to choose suitable, compatible excipients.

Differential scanning calorimetry (DSC)[6]

The DSC employed *Cardiospermum halicacabum* L. leaf extract, phospholipid, and a physical amalgamation of the pure extract and excipients. Using a DSC (Mettler) Toledo® USA) and a nitrogen environment at a warming rate of 10 °C/min, thermograms of the samples were acquired using the DSC method.

Formulation Studies

Table 1: Formulation table

Formulation Code	Extract: Phospholipid (Ratio)	Speed of rotation (rpm)
CHP-1	1:1	80
CHP-2	1:1	120
CHP-3	1:1	160
CHP-4	1:2	80
CHP-5	1:2	120
CHP-6	1:2	160
CHP-7	1:3	80
CHP-8	1:3	120
CHP-9	1:3	160

Preparation of phytosomes by rotary evaporation technique[7]

Phospholipid and the extract of C.H. were dissolved in an appropriate organic solvent, such as chloroform or methanol. The solvent was then removed under reduced pressure using a rotary evaporator, leading to the formation of a thin film. This thin film was subsequently rehydrated with an aqueous phase, such as water or a suitable buffer, to facilitate the creation of a phytosomal complex. To reduce particle size and improve homogeneity, the suspension was processed using a high-pressure homogenizer. The final phytosomal suspension was stored under controlled temperature conditions to preserve its stability.

Design of Experiment for formulation and optimization of CH-Phytosomes

Table 2: Experimental design for formulation and optimization

Factor	Levels used, actual (coded)		
	Low	**Medium**	**High**
Independent variables			
X1: Phospholipid:Extract	1:1	1:2	1:3
X2: Speed of rotation (rpm)	80	120	160
Dependent variables			
Y1: Vesicle size (nm)	Minimize		
Y2: Zeta potential (mV)	Maximize		

Characterization of phytosomes incorporated with *Cardiospermum halicacabum* L. leaf extract
Determination of Particle size and zeta potential:[8]

It is necessary to determine significant requirements including particle size and zeta potential. Malvern Zeta Sizer Nano ZS, which is designed on the principles of dynamic light scattering, was implemented to determine these parameters. One millilitre of the phytosomal formulation was dissolving in HPLC water in order to carry out the estimates. In addition, measurements were estimated with the medium viscosity of 0.8862 and a refractive index of 1.36 at a 90° scattering angle.

Transmission Electron Spectroscopy (TEM)[9]

The shape of the formulation was confirmed by transmission electron spectroscopy. TEM (Diya lab, Mumbai) was used to analyze the sample's internal structure, shape, and crystallization. It took 200 KV and a 9000x magnification to finish the experiment.

Preparation of the Carbopol gel[10]

1 gram of Carbopol 934 was dissolved in 90 ml of hot distilled water. To prepare a 1% Carbopol gel, 10 ml of glycerol was incorporated into the solution. Propyl paraben and methyl paraben were then added. The mixture was stirred until thickening occurred, and finally, a 50% w/w solution of triethanolamine was added dropwise to achieve a translucent gel.

Incorporation of CH extract loaded phytosomes in gel

The phytosomal formulation was progressively added while the Carbopol 934 gel base was carefully mixed. At the last, the phytosomal gel was stirred for 5 minutes using a mechanical stirrer.

Characterization of phytosomal gel[11]
Viscosity study

At the significant spindle and rotation the viscosity study of phytosomal gel were performed using Brookfield viscometer. In that the amount of gel were placed on surface and apply different shear stress for specific time at constant temperature and getting viscosity values with the number of runs.

Spreadability

By placing 1 g of each phytosomal gel within a circle of pre-measured diameter of 1 cm on a glass slab, placing another pre-weighed glass slab on top, and putting on a weight about 1 kg to the top glass slab for 5 minutes, the spreading of the phytosomal gel caused the change in diameter that was measured.

***In vitro* skin permeation study**

A uniform layer of CH-loaded phytosome with Rh6G was applied to the surface of goat skin. The treated skin was then placed in the Franz diffusion assembly. After an 8-hour treatment period, the skin was gently removed from the diffusion cell and thoroughly rinsed with water to remove any residual formulation. The treated portion of the skin was carefully excised and fixed in a 10% buffered solution. After fixation, the skin specimen was embedded in paraffin wax. Using a microtome, sections of 4.5 micrometres in thickness were cut perpendicular to the skin surface. These thin tissue sections were then placed on glass slides for examination using fluorescence microscopy.

***In vitro* anticancer activity**

Cytotoxicity[12]

Cells were seeded in a 96-well flat-bottom microplate and incubated at 37°C with 95% humidity and 5% CO2 overnight. Various concentrations of the sample (100, 50, 25, 12.5, 6.25, and 3.125 µg/ml) were then applied to the cells, and they were further incubated for 48 hours. After incubation, the wells were washed twice with PBS. To each well, 20 µL of MTT staining solution was added, followed by incubation at 37°C for 4 hours. After the incubation period, 100 µL of DMSO was added to dissolve the formazan crystals. The absorbance was then measured at 570 nm using a microplate reader.

Apoptosis by Flowcytometer[13]

Cells were seeded in a 6-well flat-bottom microplate containing cover slips and incubated overnight at 37°C in a CO2 incubator. After 24 hours, the GI50 concentration of each sample was applied to the cells. Following the incubation, the cells were washed twice with PBS and then centrifuged at 500×g for 5 minutes at 4°C. The supernatant was discarded, and the cell pellets were resuspended in ice-cold 1X Binding Buffer, adjusting the concentration to 1 × 10^6 cells per ml. The tubes were kept on ice. To each tube, 5 µL of Annexin V AbFluor 488 and 2 µL of the appropriate reagent were added. The mixture was gently mixed and kept on ice. The cells were incubated in the dark for 15 minutes. After incubation, 400 µL of ice-cold 1X Binding Buffer was added, and the solution was gently mixed. Analyse cell preparation within 30 minutes using flow cytometry. Perform analysis using FlowJoX 10.0.7softwere.

Live and dead cell assay[13]

Cells were seeded in a 24-well flat-bottom microplate containing a coverslip and maintained at 37°C in a CO2 incubator overnight. After 48 hours, 200 µL/mL of the compound was applied to the cells. After incubation, the cells were washed with PBS and then fixed with 4% paraformaldehyde for 30 minutes. A mixture of Acridine orange and ethidium bromide (20 µL) was applied to the cells, which were incubated for 5 minutes at room temperature in the dark. Finally, the cells were examined under a fluorescence microscope.

Stability

To assess the long-term stability of the *Cardiospermum halicacabum* leaf extract-loaded phytosomal gel formulation, samples were stored at room temperature and 4°C (±2°C) for 45 days. The formulation was monitored every 15 days for pH, spreadability, and physical appearance, checking for phase separation, color changes, or texture alterations. This study evaluates the formulation's stability and provides important data on its shelf-life and potential pharmaceutical or cosmetic applications.

RESULTS AND DISCUSSIONS

Compatibility study using FTIR

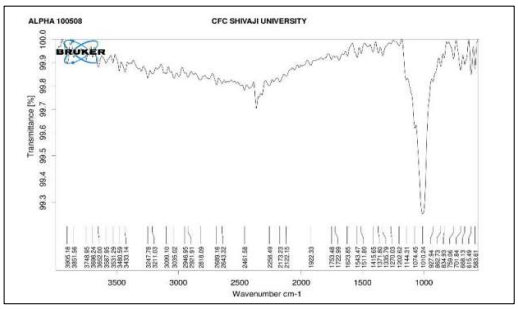


Fig 1: FT-IR of extract

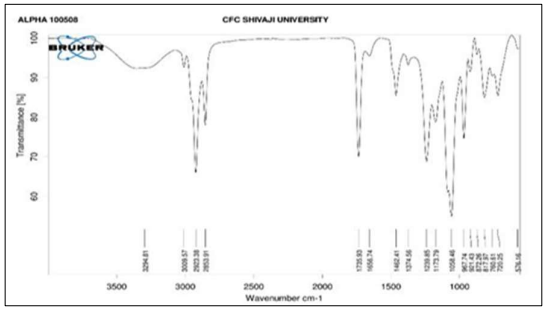


Fig 2: FT-IR of Phospholipid

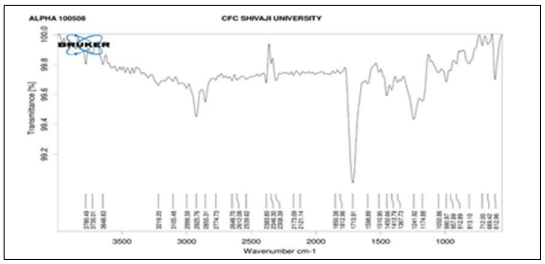


Fig 3: FT-IR of Physical mixture

Differential Scanning Calorimetry (DSC)

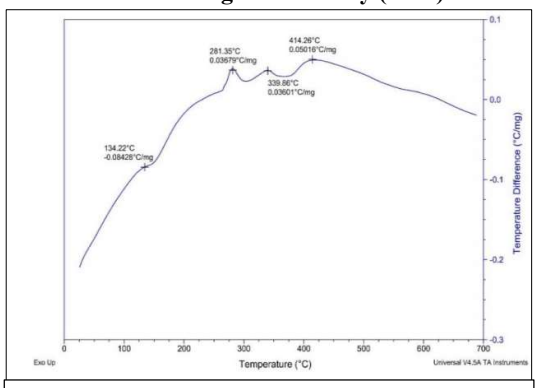


Fig 4: DSC of extract

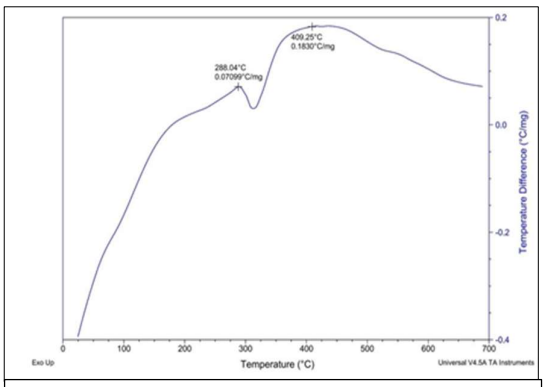


Fig 5: DSC of Phospholipid

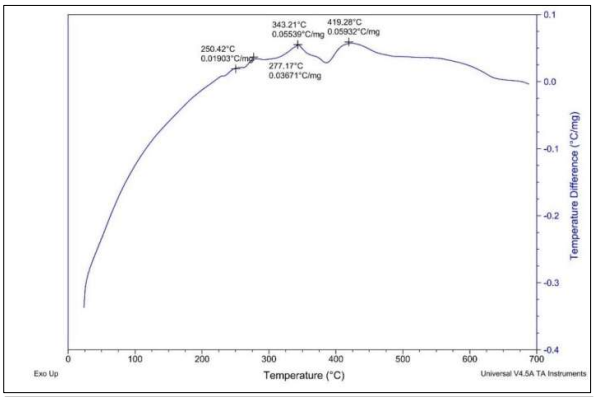


Fig 6: DSC of Physical mixture

Experimental design

ANOVA for Linear model: Response 1: Vesicle size

Effect of phospholipid and speed of rotation on vesicle size

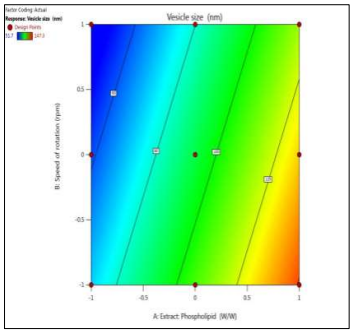


Fig 8: size3D Surface graph of vesicle size

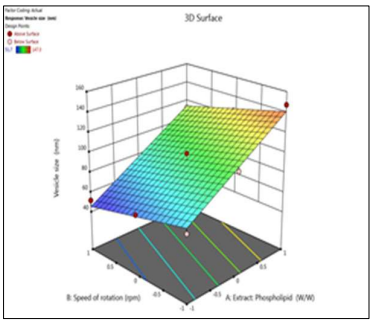
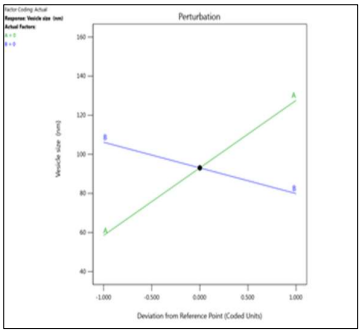


Fig 9: Perturbation graph of vesicle size



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Response 2: Zeta potential

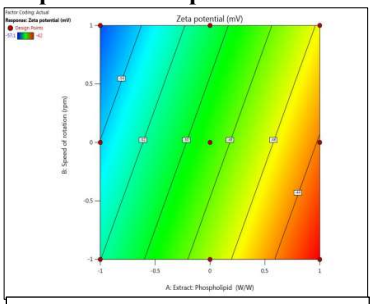


Fig 10: 2D Response Surface graph

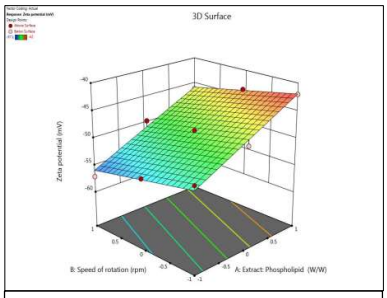


Fig 11: 3D Response Surface graph

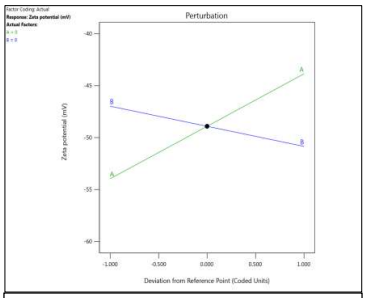


Fig 12: Perturbation Surface graph of zeta

Characterization of Phytosomes: Determination of particle size

Particles in the produced phytosomes range in size from 51.7 nm to 147.3 nm. The outcomes of the particle size analysis presented in when the quantity of *Cardiospermum halicacabum* L. leaf extract was constant were Table show that decreasing the phospholipid concentration from 1:3 to 1:1 significantly decreased the particle's size. Thus, only a small amount of phospholipid is needed to create microscopic phytosomes**.**

Table 3: Particle size of phytosomes

Formulation code	Particle size (nm)
CHP -1	66.4
CHP -2	60.1
CHP -3	51.7
CHP -4	101.8
CHP -5	99.6
CHP -6	72.4
CHP -7	147.3
CHP -8	125.6
CHP -9	112.6

Determination of Zeta potential

The zeta potential characterization of CH loaded phytosomes yielded a range of results, spanning from -42.0 mV to -57.1 mV. s. A zeta potential within this range suggests a strong surface charge, indicative of good dispersion stability and potential resistance to agglomeration. The negative values imply the presence of anionic charges, which may contribute to repulsive forces between particles, further enhancing stability.

Table 4: Zeta potential of phytosomes

Formulation code	Zeta potential (Mv)
CHP-1	-51.2
CHP-2	-53.8
CHP-3	-57.4
CHP-4	-48.1
CHP-5	-48.5
CHP-6	-49.7
CHP-7	-42.0
CHP-8	-43.7
CHP-9	-46.1

Morphology of phytosomes through Transmission Electron Microscopy (TEM)

The CH-P 3 formulation was chosen as the most optimal, consequently undergoing TEM analysis to capture the phytosome's imagery, complete with a specified scale bar and the indicated magnification level. The phytosomes exhibited nearly spherical shapes and featured a notably smooth surface on the lipid bilayer.

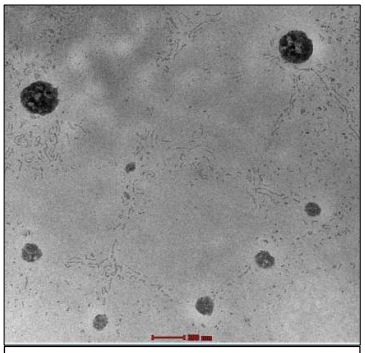


Fig 13: TEM of CHP-3 Formulation

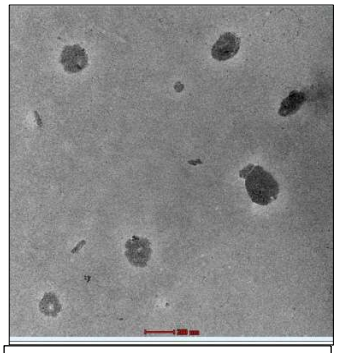


Fig 13.1: TEM of CHP-3 Formulation

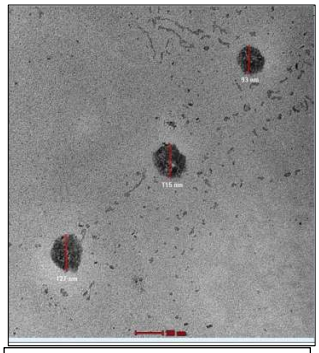


Fig 13.2: TEM of CHP-3 Formulation

Characterization of phytosomal gel

Viscosity study

The utilization of a Brookfield viscometer revealed that the phytosomal gel exhibited a viscosity of 12.5175 Pascal seconds (Pa*s).

Spreadability study

The spreadability of the phytosomal gel was found to be 7.4 ± 0.26 cm. This data on spreadability clearly demonstrates that the phytosomal gel performed exceptionally well, yielding the most favorable results.

pH determination

The pH of phytosomal gel of CH-P 3 batch was found to be 6.5 ±0.01.

***In vitro* skin permeation study**

To effectively treat melanoma, it is crucial for the drug to penetrate into the deeper layers of the skin, as melanoma originates from melanocytes located in the lower layers of the skin's epidermis. Upon examining fluorescence microscope images of goat skin after a 6-hour treatment with a rhodamine-6G solution (control) and Rh6G-CHP, notable differences were observed. The Rh6G solution exhibited fluorescence limited to the superficial stratum corneum (SC) layer. In contrast, treatment with Rh6GCHP resulted in a relatively uniform and intense fluorescence spanning the SC, epidermis, and dermis layers. This substantial increase in fluorescence intensity from the deeper layers of the skin signifies the successful delivery of phytosomes to these regions. It demonstrates the phytosomes' remarkable ability to enhance skin penetration and facilitate the transportation of active agents deeply into the epidermis and dermis, which is essential for effective melanoma treatment.

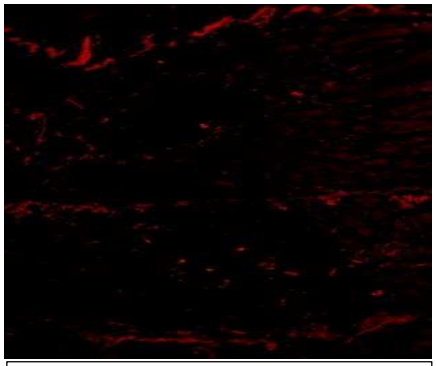


Fig 14: *In vitro* skin permeation study effects

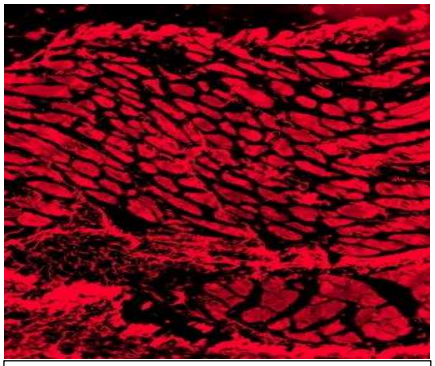


Fig 15: *In vitro* skin permeation study effects

***In-vitro* anticancer activity**

Cytotoxicity

The study assessed the cytotoxic effects of CH-P gel on MCF-7 (Breast cancer) and A431 (Melanoma) cells using the MTT Assay. The results were compared with those obtained using *Cardiospermum halicacabum* It was observed that the viability of MCF-7 and A431 cells showed concentration-dependent cytotoxicity after 48

hours of incubation. The CH-P gel demonstrated greater cytotoxicity (low IC50: 59.36 µg/ml) compared to C.H. (91.37 µg/ml) against MCF-7 cells. Similarly, against A431 cells, the CH-P gel exhibited significant cytotoxicity (IC50: 31.69 µg/ml) compared to C.H. ($p<0.01$), prompting further studies on the A431 cell line.

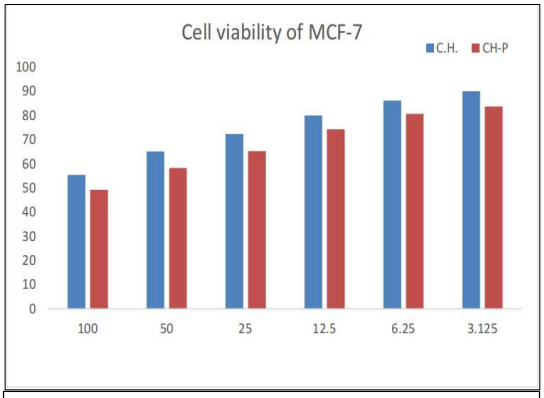


Fig 16: Cytotoxicity study on MCF-7 cell line

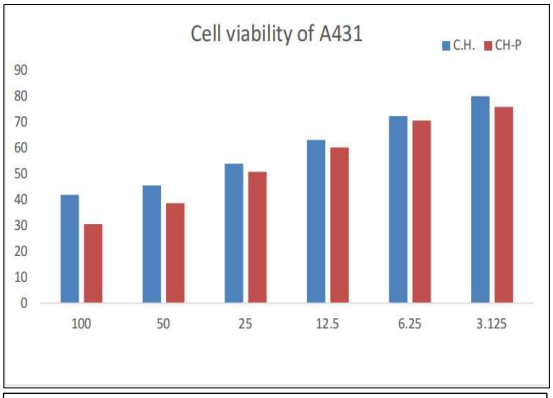


Fig 17: Cytotoxicity study on A431 cell

Apoptosis by Flow Cytometry

The cytometric analysis of A431 cells subjected to CH and CH-P gel treatments is presented in Figure 7.18 (B) and 7.18 (C). In the control group, untreated cells exhibited predominantly healthy characteristics (98.7% viability), with only 0.61%, 0.44%, and 0.28% of cells in early, late apoptotic, and deceased states, respectively (Figure 7.18 (A)). Consequently, the percentage of viable cells experienced a rapid decline to 83.9% upon exposure to CH treatment (Figure 7.18 (B)). In contrast, cells treated with CH-P gel displayed only 24.1% and 0.95% of cells in early and late apoptotic states, respectively. As a result, the proportion of viable cells exhibited a significant reduction to 74.6% when treated with CH-P gel (Figure 7.18 (C)).

Table 5: Apoptotic values

Sl.NO	Treatments	Live		Early Apoptosis		Late Apoptosis		Dead	
		1st	2nd	1st	2nd	1st	2nd	1st	2nd
1	NC	98.7	97.4	0.61	0.92	0.44	0.66	0.28	0.96
2	Cardiospermum halicacabum	83.9	84.6	15	14.3	0.88	0.95	0.54	0.21
3	C.H.loaded phytosomes	74.6	72.8	24.1	25.6	0.95	1.3	0.28	0.35

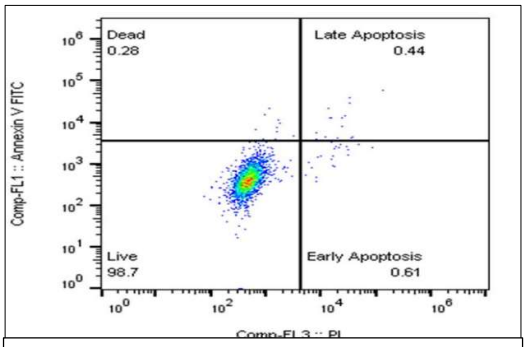


Fig 18: Normal control A431 Melanoma cell line

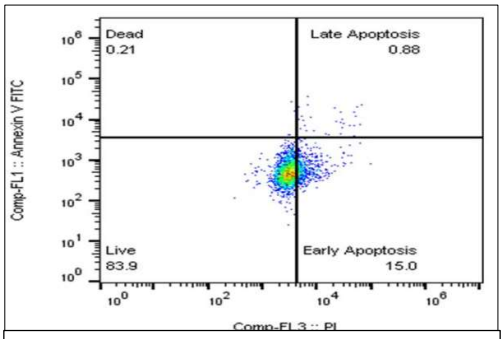


Fig 19: *Cardiospermum halicacabum* L. extract treated A431 Melanoma cell line

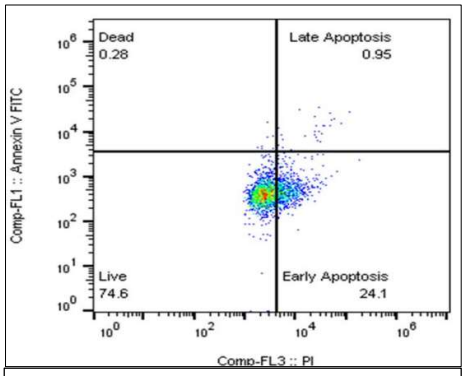


Fig 20: CHP-3 gel treated A431 Melanoma cell line treated A431 Melanoma cell line

Live and dead cell assay:

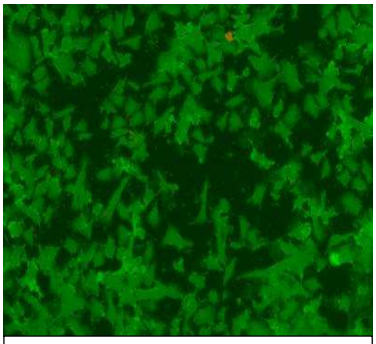
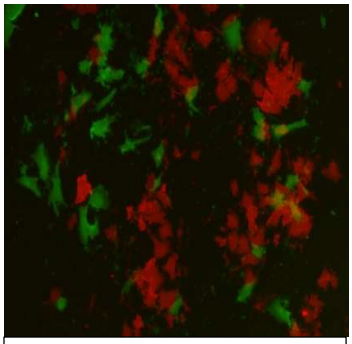
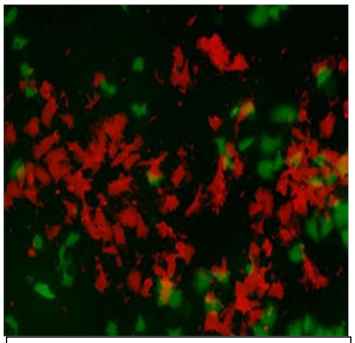


Fig 21: Normal control A431 Melanoma cell line



(B): *Cardiospermum halicacabum* L. extract treated A431 Melanoma cell line



(C): CHP-3 gel treated A431 Melanoma cell line

This test employs a blend of two intensely fluorescent dyes, AO and EB, to distinguish between living and deceased cells. AO infiltrates living cells with undamaged membranes, producing a vivid and steady green fluorescence. On the other hand, EB can only penetrate deceased cells with compromised membranes, producing a vivid orange or red fluorescence. Fluorescence microscopy images depict the live and dead cell assay results for CH and CH-Phytosomes in the A431 cell line following a 24-hour incubation period. Cells subjected to CH and CH-P treatments displayed a distinct dark orange-red coloration. Live cells were characterized by vibrant green fluorescence, while deceased cells exhibited a noticeable dark-orange red fluorescence. The demise of the cell's plasma membrane, induced by CHP Upon completion of the treatment period, might be the underlying factor for cell demise.

Stability

The stability of formulation was tested in 4°c ±2 and at the room temperature. The subjected samples showed pH and spreadability changes within the limit up to the testing duration of 45 days with acceptable slight change. Overall, the stability study indicates that the CH loaded phytosomal gel maintains satisfactory stability in terms of both pH and spreadability under the specified storage conditions

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