Efficacy of Concanavalin-A conjugated nanotransfersomal gel of apigenin for enhanced targeted delivery of UV induced skin malignant melanoma

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ABSTRACT
The aim of present study was to develop the efficient targeting of Concanavalin-A conjugated nanotransferosomal gel to bind directly to melanocytes gel layer against UVB induced skin carcinoma. Carbopol loaded nanotransferosomal gel have prepared by modified rotary evaporation sonication technique & conjugated synthesized by carbodiimide method and they were characterized the morphology, zeta potential, penetration and cell viability. In vitro release studies & skin permeation have determined using Franz diffusion cell and confocal laser scanning microscope (CLSM). The conjugated formulation showed vesicles size, polydispersity index, zeta potential and % conjugation efficiency of 179.0 ± 0.32 nm, 0.197 ± 0.07, 35.1 ± 0.21 mV and 89.73 ± 1.29% respectively. The surface morphology was confirmed by transmission electron microscopy (TEM) and FTIR to make sure the compatibility among its ingredients. Con-A conjugated nanotransferosomal gel showed toxicity on melanoma (A375) in a concentration range of 0.4–2.0 mg/mL, but less toxicity toward HaCaT cells. The MTT assay has analyzed against two different cell lines, to determine their anti-cancer potentials and their targeting ability. Conjugated formulation were found to decrease the cell viability, higher skin targeting efficacy in in-vitro & in-vivo. Concanavalin conjugated nanotransferosomal gel of apigenin promise an efficient and economic approach for the skin cancer.

Introduction
Natural Drugs targeting specific organs and tissues has become one of the critical endeavours and significant challenges of the current era along with improving therapeutic index and bioavailability at site-specific delivery. The clinically significant impact of targeted drug delivery lies in the ability to specifically target a drug or drug carrier to minimize drug-originated systemic toxic effects. Targeted drug delivery systems have been developed to optimize regenerative techniques [1]. Although conventional chemotherapy has been successful to an extent, the main drawbacks of chemotherapy are its poor bioavailability, high-dose requirements, adverse side effects and low therapeutic indices, which is being faced by the researchers in the anti-cancer formulation. We have exhibited that the enhanced permeability and retention is the basis for using nanobioconjugated active targeting formulation, aiming for a better understanding of tumour biology in the current years and new targeted drug delivery approaches that are being explored for effective dermal delivery at the targeted or specific site is significant. The advantages of the targeted release system are the reduction in the frequency of the dosages taken by the patient, having a more uniform effect of the drug, reduction of drug side-effects, and reduced fluctuation in circulating drug levels [2–4]. Thus, bioconjugates nanocarriers therapy, worldwide, is to be used progressively for targeted delivery and has a high expansion treatment efficacy for melanoma skin cancer.

Lectins, a group of specific glycoproteins present in the animals as well as plant cells, are extracted from jack bean seeds. Although lectins have been widely studied for their potential in skin cancer diagnosis and have also been investigated for significant antiproliferative activities both in-vitro and in-vivo and generation from diverse mechanisms, including apoptosis, autophagy, and inhibition of tumour growth [5]. Con-A is one of the most abundant lectins for a potential anti-neoplastic agent for melanoma cancer therapeutics and to prevent their spread [6]. Several studies have demonstrated that Con-A induces caspase-dependent apoptosis in melanoma A375, have the strength to induce autophagy in hepatoocyte cell, and inhibits proliferation on B16 melanoma cells and fibroblast 3T3 cells [7–9].

Malignant melanoma skin cancer mainly occurs due to UV-light exposure because a large amount of UV radiation reaches earth’s surface, attributable to the slow depletion of the ozone layer. The increases of UVB (290 to 320 nm) directly damages the cellular DNA leading to the formation of 6-4 cyclobutane pyrimidine dimmers, DNA base and nucleoprotein crosslinking, and polynucleotide chains

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disruption [10]. UVA (320 to 400 nm) indirectly damages the DNA by the production of oxygen reaction species. This type of skin cancer is associated with the melanocytes of the epidermal layer. UV radiation can cause the suppression of the immune system that leads to inadequate prevention of cancer formation [11,12].

Despite the recent advancement in therapeutics, significant challenges are yet present in the field of skin cancer. Commonly used chemotherapy has given unsatisfactory results. Many drugs like carmustine, dacarbazine, paclitaxel, cisplatin and carboplatin can add to the side effects as hair loss, loss of appetite, diarrhea, fatigue and increased risk of infection, as the therapy is deleterious to patient health by making patients more susceptible to other diseases and often cause death by weakening the immune system of the patient [13].

Nanocarriers have been demonstrated to increase the solubility and bioavailability of poorly soluble-in-water drugs. Nanocrystals and mixed micelles system, which are not only promising and effective for improving aqueous solubility of apigenin but also have the ability for controlled drug release and precise targeting have been reported previously [14,15]. Nanovesicular carriers have been used to deliver drugs to target sites for cancer therapeutics. These lipid nanocarriers are adsorbed into the skin surface, allowing lipid exchange between the outermost layers of the stratum corneum [16].

Recently, the strength of bioactive medicine to suppress carcinogenesis has attracted the worldwide attention of cancer exploration and treatment, among researchers. Apigenin is a bioflavone compound, which is present in a wide variety of plants and herbs and distributed plentifully in common vegetables and fruits such as grapes, apples, garlic, onions, oranges, chamomile tea and parsley [17]. Apigenin that is significant chemically (4,5,7-trihydroxyflavone) is a promising skin cancer chemotherapeutic agent, both in-vitro and in-vivo, which are serving diverse physiological functions, for example, as strong anti-inflammatory, antioxidant, anti-carcinogenic, antiviral activities, anti-aging, antibacterial and blood pressure reduction [18]. Apigenin have been previously studied for human cancers in vitro and in vivo by multiple biological effects such as inducing cell cycle arrest, autophagy, triggering cell apoptosis, cell migration, invasion and suppress immune response [19,20]. Apigenin has also been studied to suppress protein kinase cell activity and prevention of UVB-induced skin carcinogenesis and most type of cancers, including melanoma, breast cancer, lung cancer, colorectal cancer, prostate cancer, osteosarcoma and liver cancer [21,22].

More studies have shown that advanced liposomes, niosomes of natural medicine have limited skin benefit, production instability and low bioavailability of compounds exhibiting antitumorogenic, antioxidants and anti-inflammatory effects [23–25] (reference. Kotla et al. [26] have reported that liposomal drug delivery is unsuccessful due to their inability to permeate the deeper layers of the skin, which showed a lack of prospective availability as a carrier and reservoir for sustaining and degrading permeability within wide layers of the skin. Among them, polyphenol loaded nanotransfersomes have good strength to improve baicalin efficacy in inflammatory skin disorders and also phospholipids vesicles based hydrogels have improved in dermal delivery and formulation stability [27,28]. Transfersomes are self-regulating and self-optimizing ultradisperseable liposomes nanovesicles and are composed of water, surfactants, and phospholipids for enhanced and significant chemotherapeutic dermal drug delivery and as a drug carrier for non-invasive targeted drug delivery and sustained release of therapeutic agents [29,30].

Presently, the most promising aspect of utilization of various bioconjugates phytomedicine has been reported by different researchers and scientists such as Saraf et al. [31], who have reported that PEGylated nanoparticles accumulate in tumours by the enhanced permeation and retention (EPR) effect as a function of cellular adhesion. Developing truly targeted drug delivery systems has to be based on a clear understanding of the problems that must be solved. Folate receptor-fabricated nanogels can serve as a better option for treating various cancers in terms of drug loading and stability, and cellular uptake of anticancer drugs studied and performed by Gupta et al. [32] was significant. Con-A conjugated nanotransfersomal gel was employed as the nanocarrier to enhance cancer cell targeting, Con-A as targeting ligands, carbopol as a linker for prolonging circulation and phospholipids for flexibility on the skin surface. Our objective was to utilize con-A conjugated nanotransfersomal gel of a natural anticancer drug to investigate its improving anticancer efficacy and enhanced targeted delivery of UV induced skin malignant melanoma and reducing the systemic toxicity commonly accompanying chemotherapeutics.

Materials and Methods

Materials

Apigenin was purchased from Sigma Aldrich (Bombay). Tween 80, Rhodamine, [1-Ethyl-3,3-(dimethylaminopropyl) carbodiimide] (EDC) and Phospholipid were purchased from Himedia Pvt, Ltd. Mumbai, India. Phospholipid (phosphotidylcholine) that is present in soy lecithin containing not less than 98% PC and Florida cream, were purchased from Shalak Pharmaceutical Pvt, Ltd. Mumbai, India. Phospholipid (phosphotidylcholine) was purchased from Bio-tech pharmaceutical. Apigenin is a gift sample by Bio-research products, Inc., 323W. Cherry St., North library (IA 5231), N-Hydroxysuccinimide (NHS) was supplied as a gift sample from Shivam Enterprises, Pune, Maharashtra (India). Purified water from Millipore ultrapure water system (Synergy UV water purifier system, India) was used throughout the study. All other chemicals used in the study were of analytical grade.

Chemicals for cell lines and culture

The two cell lines were obtained from the National Center for Cell Science, Pune, India. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Aldrich Ltd, India, HaCaT cells were cultured in Dulbecco’s Modified Eagle’s Medium Nutrient Mixture F-12 HAM (DMEM F-12 HAM) with 2 mM L-glutamine supplemented with 10% fetal bovine serum (FBS). Penicillin (100 mg/mL), streptomycin (100 mg/mL) and amphotericin-B (5 mg/mL), and the cells were incubated in CO₂ incubator.
with 5% CO₂. The cell suspension was centrifuged at 3000 rpm for 3 min and then re-suspended in the growth medium for further studies.

**Methods**

**Preparation of nanotransfersomes by rotary evaporation sonication technique**

Apigenin loaded nanotransfersomes were formulated by a rotary evaporation sonication technique with slight modifications as demonstrated by Cevc et al. [33] and Saraf et al. [32]. Concisely, apigenin drug, tween 80 and soya phosphotidylcholine were dissolved in ethanol. Ethanol was evaporated by rotary evaporation (Buchi rotavapor R-3000 with 14,000 Water bath, Switzerland) under reduced pressure above the lipid transition temperature for the formation of lipid film on the wall of the flask. The film was hydrated at 45 °C using a saline phosphate buffer solution (pH 5.5) by rotation at 60 rpm [29,34]. The transfersomes formed were allowed to swell for 2 h at room temperature. The resulting vesicles were sonicated for 30 min in a probe sonicator to reduce the size of the vesicles uniformly. The prepared transfersomes were stored in an airtight container for further study.

**Conjugation of Con-A with apigenin loaded nanotransfersomes**

Apigenin-loaded nanotransfersomes were Con-A conjugated using Carbodiimide method with some modifications as reported by Damink et al. [35] and Jangdey et al. [36]. Briefly, activation of carboxyl group of nanotransfersomes (50 mL) was carried out by addition of 1 mL of 0.1 M 1-ethyl-3,3-(dimethylaminopropyl) carbodiimide (EDC) and 1 mL of 0.11 M of N-hydroxysuccinimide (NHS) in phosphate buffer (pH 5.5). After 3 h incubation at room temperature, excess coupling agent and byproducts were removed by washing nanotransfersomes with phosphate buffer (pH 5.5) and filtered. The latter was suspended in 10 mL of Con-A solution in phosphate buffer (pH5.5) and after incubation for overnight. Con-A conjugated nanotransfersomes were obtained by centrifugation (C-24, BC, Cooling centrifuge, REMI, India) for 30 min at 9000 rpm, followed by three to four times washing with Millipore water. The obtained Con-A conjugated nanotransfersomes thus were dried at room temperature.

**Preparation of conjugated nanotransfersomal based Gel**

Conjugated nanotransfersomal base gel was prepared as previously described by Khan et al. [37], with some modified, increased apigenin loading. Briefly, dispersing the 1 g of the Carbopol 934 in a sufficient quantity of Millipore distilled water. After complete dispersion, the Carbopol 934 solution was kept in the dark for 24 h for complete swelling. Afterwards, the optimized conjugated nanotransfersomes was gradually added to the viscous solution of Carbomer 934 beneath magnetic stirring. The pH values were subsequently regulated to 5–6. Then other ingredients like isopropyl alcohol (5% wt/wt), PEG-400(5% wt/wt), PG (5% wt/wt), and triethanolamine (0.5g) were added to obtain a homogeneous dispersion of gel.

**Characterization of Con-A conjugated nanotransfersomes**

**Morphology, vesicle size and Zeta potential**

The surface of conjugated nanovesicle transfersomes was selected and explored by transmission electron microscope (TEM), (Hitachi J 500, H7500 Japan). One drop of sample Con-A conjugated nanotransfersomes was placed on carbon-coated grids followed by negative staining with 2% w/v aqueous solution of phosphor-tungstic acid for viewed visualization of vesicles at 100 kV.

Size of the nanotransfersomes and Con-A conjugated nanotransfersomes was analyzed by dynamic light scattering (DLS) with a Malvern Zetasizer 3000 HAS (Malvern Instruments, Malvern, UK). The formulation (0.1 mL) was dispersed in 50 mL of sufficient amount of ultra-purified water in a volumetric flask and mixed thoroughly with vigorous shaking before the conduct of experiment and light scattering was monitored at 25 °C at a 90° angle between laser and detector.

Measurement of the zeta potential for Con-A conjugated nanotransfersomes and nanotransfersomes was determined using a Zetasizer (Malvern instrument, USA). Samples were placed in clear disposable zeta cells and results were recorded. The sample was diluted by Millipore ultra-purified water prior to the experiment. The measurements were performed in triplicate [12].

**X-ray diffraction study**

The analysis of the natural crystalline or amorphous of apigenin-loaded nanolipid Con-A conjugated transfersomes was performed by X-ray diffraction (XRD). X-ray powder diffraction studies of pure drug apigenin, phospholipid (soya lecithin), Con-A, physical mixture (apigenin, phospholipid and Con-A) in ratio of (1:1:1) and Con-A conjugated nanotransfersomes were carried out using powder X-ray diffractometer (PANalytical 3kWX’ pert powder, Cambridge, UK). Samples were positioned in the sample stage and scanned from 2° to 60° with an operating voltage of 40 kV and current of 30 mA. A Cu-Ka radiation source was used and the scanning rate 2 h/min was 5 °C/min.

**FTIR study**

FTIR spectrum (Shimadzu 8400U) was carried out to confirm the conjugation of Con-A with apigenin loaded nanotransfersomes. Drug sample (apigenin, Con-A and Con-A conjugated nanotransfersomes) was analyzed using the KBr pellet technique. ~1–5 mg of the sample was mixed with dry potassium bromide and the samples were examined at transmission mode over wavenumber range of 4000 to 400 cm⁻¹.
**Differential scanning colorimetry study**

Differential Scanning Colorimetry (DSC) is one of the thermo-analytical techniques for assessment of the heat energy uptake for providing data on endothermic (heat absorption) and exothermic (heat evolution) processes. DSC analysis was performed using a DSC instrument (Mettler 305, Switzerland). The sample of 4 mg was placed in aluminium pans (Al-Crucibles, 40μl Al) and scanned between 25 to 400 °C under a nitrogen atmosphere and at a heat flow rate of 10 °C/min. The samples were studied for pure drug, phospholipids, Con-A, carbodiimide and Con-A conjugated nanotransfersomes.

**Determination of interference of formulation additives in the estimation of apigenin**

Stock solution of apigenin (100 μg/ml) in methanol was placed separately in five volumetric flasks. To this solution, 10 mg of each additive was added in separate volumetric flasks. The flasks were manually stirred for 6 h and kept overnight. The solution of each flask was filtered by Whatman filter paper (#41). Each solution was diluted sufficiently to produce the final concentration of 5 μg/ml in separate volumetric flasks. The absorbance of resultant solution was measured at 336 nm using UV-visible spectrophotometer (Systronics, Japan).

**Conjugation efficiency of Con-A nanotransfersomes and percent entrapment efficiency**

Apigenin loaded nanotransfersomes were bound to Con-A and the amount of Con-A was calculated as the difference between the Con-A added initially and the Con-A recovered after incubation with the nanotransfersomes. The amount of lectin in the supernatant was estimated by using Folin–Ciocalteu phenol reagent. Reagent C (10 mL) was added in a suspension containing 50 mL of conjugated nanotransfersomes in 50 mL of millipore distilled water, mixed thoroughly and allowed to stand for 20 min. Then 1 mL of the reagent D was added rapidly with immediate mixing to the above solution. After 30 min, the solution was filtered with Whatman filter paper (#41), volume was measured against blank using a UV-visible spectrophotometer (Perkin Elmer Lambda 25 double beam UV-Visible Spectrophotometer, Singapore) at 750 nm. The experiment was performed in triplicate. Reagent A and reagent B were prepared by dissolving Na2CO3 (2 g) in 0.1 N NaOH and CuSO4·5H2O (0.5 g) in 1% sodium/potassium tartrate to make 100 mL solutions each, respectively. Reagent C was prepared by mixing 50 mL of the reagent A with 1 mL of reagent B. Reagent D was prepared by dissolving Folin–Ciocalteu phenol reagent (2.0 N) with distilled water to make it 1.0 N [36].

The quantity of apigenin entrapped within Con-A conjugated nanotransfersomal gel was determined by ultracentrifugation. The unentrapped apigenin in nanotransfersomes dispersion was separated by cooling centrifuge (Remi instruments Ltd., Mumbai, India) at 13,000 rpm, for 20 min at room temperature and the supernatant was decanted. The concentration of apigenin in the supernatant was determined using UV-visible spectrophotometer at \( \lambda_{\text{max}} \) 336 nm. Each experiment was performed in triplicate [38]. The % entrapment efficiency was calculated with the following equation:

\[
\% \text{Entrapment efficiency} = \frac{\text{Calculated drug content}}{\text{Theoretical drug content}} \times 100
\]

(1)

**Spreadability**

Spreadability measurements of all the developed gels were carried out individually in triplicate using a wooden block and glass slide apparatus. A modified apparatus consisting of two glass slides containing sample in between, with the lower side fixed to a wooden plate and upper one attached to a balance by a hook, was used to determine the Spreadability. Specified amount of gel was taken, and the spreadability was calculated using the following formula:

\[
S = M \times \frac{L}{t}
\]

(2)

Where \( S \) = Spreadability in g cm s⁻¹; \( M \) = Mass of gel placed between the two slides; \( L \) = Length of slide; \( t \) = Time taken by the upper slide to detach [39].

**In vitro drug release study**

In vitro release studies of drug loaded Concanavalin conjugated nanotransfersomes and pure drug suspension and marketed product was carried out for each drug individually using modified Franz diffusion (Mol. Wt. cut of 6000–8000, Hi Media Ltd, Mumbai, India). Vertical Franz Diffusion cell was designed and validated prior to the release study. The cellophane membrane was mounted on a diffusion cell assembly having a diffusion area of 2.5 cm. The receptor compartment consisted of a 22.5 mL phosphate buffer at pH 5.5 as the receptor fluid agitated at 100 rpm, and was maintained at 37 ± 0.5 °C throughout the experiments. The prepared formulation was placed in the membrane of the donor compartment. An aliquot of 2 mL sample was withdrawn at suitable time intervals and replaced immediately with an equal volume of fresh diffusion medium. The % drug release was calculated and a graph of % drug release against time was plotted, release studies were performed in triplicate for each formulation [40]. Data were expressed as the cumulative amount of apigenin permeated through the membrane considering the total amount of drug applied of each formulation.

**Release kinetics analysis**

Release kinetics from the Con-A conjugated nanotransfersomal gel formulation were compared to different kinetic models. The models studied were: Zero order rate kinetics, first order rate kinetics, Higuchi’s kinetics. In this kinetic behaviour, the graph is plotted between the cumulative percent release with respect to time. In Ritger–Peppas exponential kinetics, the graph is between the log of cumulative percent drug release with respect to time [41].
**In-vitro skin retention and deposition study**

The skin deposition and retention studies of various formulations were conducted in order to analyze the content of apigenin in the skin after 24 h of diffusion. At the end of the experiment, the skin mounted on the diffusion cell was removed safely, and the skin surface samples were washed up with distilling and methanol on both sides and carefully dried. For the assessment of drug deposition and retention in the skin, the cleaned skin was cut into small pieces, mashed completely in a beaker and 10 mL of 50% methanol was added to this mass. Finally, the beaker was mechanically shaken in a water bath shaker at 37 °C for 3 h for the complete isolation of the drug. After suitable dilution, the apigenin content in the filtrate was determined by UV spectroscopy [42].

**Ex-vivo skin permeation studies**

**Goatskin samples**

Fresh Goatskin was collected from a local slaughterhouse. After thorough washing, the hair on the skin was removed using a surgical razor and the skin was separated from the underlying cartilage and subcutaneous fat using a scalpel and cut into appropriate sizes. The skin was stored in a formalin solution at 4 °C. The skin was first immersed in ultra-purified water at 60 °C for 2 min and the epidermis was then peeled off. Dried skin samples were kept under –20 °C for later use.

**Assessment of permeation studies**

The ex-vivo skin permeation analysis was performed to use the full-thickness goat skin through using modified fabricated Franz diffusion cell. The skin was cut with an effective diffusion area of 2.5 cm² and clamped between the donor and the receptor chamber of diffusion cell. The receptor chamber was filled with freshly prepared phosphate buffer pH 5.5. The diffusion cell was maintained at 37 °C and the solution of the receptor chamber was stirred continuously at 500 rpm by using magnetic stirrer with a hot plate (Remi equipments, Mumbai). The formulation (2 ml) was gently placed in the donor chamber at 1, 2, 3, 4, 5, 6, 12, 18 and 24 h, 5.0 mL of the solution in the receptor compartment was removed and analyzed using UV-spectrophotometer and replaced immediately with an equal volume of fresh buffer. The permeation coefficient of goot skin (Kp) was calculated using the equation $K_p = J_{sp}/C$, where C is the initial concentration of the drug in the formulation pertain to the donor phase [37].

**Histopathological evaluation of skin for photodamage**

The animal skin was constantly examined for 6 weeks for the slow progress of photocarcinoma. The evaluation criterion was adopted from Kaur et al. [32] with slight modifications. An evaluatory scale was developed according to the skin conditions from 0–5, with representations of normal skin at zero and severely damaged skin at 5. Further skin biopsies were taken every week from each group of animals and were embedded in paraffin after fixing in 10% buffered formalin. Then the sections were sliced with a semi Automated Rotary Microtome (Leica®, Germany) and stained accordingly using Hematoxylin and Eosin (H and E) staining dyes. Finally, the stained specimens were observed under a Fluorescence microscope (Nikon®, India), in order to determine the extent of damage caused to the skin components [43].

**Myeloperoxidase assay**

MPO is an enzyme which generates reactive oxidant species as part of its function in innate most defence mechanisms and critically expressed in UV irradiated skin. In brief, a snap frozen skins biopsy of size about 10–20 mm was harvested and incubated in hexadecyltrimethylammonium bromide containing phosphate buffer at a temperature of −80 °C. After incubation, the tissue samples were subjected to homogenization followed by sonication and centrifugation. Centrifugation helps in removing skin debris. The supernatant liquid left after centrifugation was analyzed spectrophotometrically for myeloperoxidase levels. A comparison of exposed tissue samples readings was done with the control group in order to correlate the results with the release profile of myeloperoxidase levels and their inhibition by the prepared formulations [44].

**Confocal cell uptake studies**

The slides of skin penetration of Rhodamine 123 were then viewed under confocal laser scanning microscopy (CLSM) to study the internalization of apigenin loaded Con-A conjugated nanotransfersomal gel by the tissues at Osmania University, Hyderabad. After applying Goatskin, in vitro, to systems containing probe and Conjugated nanotransfersomes containing 0.03% rhodamine, they were used for the assessments. The conjugated nanotransfersomal gel was applied homogeneously and nonocclusively to skin tissue. The experiments were run in Franz diffusion cells and the receiver contained phosphate buffer pH 5.5 solution. After 24 h, the skin was removed and washed with phosphate buffer. The skin was then rapidly frozen by liquid nitrogen and a skin surface perpendicular rectangular piece was taken from the site of drug application with the help of a sharp blade. This skin tissue was fixed on the sample holder with the help of a Tissue puncher and critically expressed in UV irradiated skin. In brief, a snap frozen skins biopsy of size about 10–20 mm was harvested and incubated in hexadecyltrimethylammonium bromide containing phosphate buffer at a temperature of −80 °C. After incubation, the tissue samples were subjected to homogenization followed by sonication and centrifugation. Centrifugation helps in removing skin debris. The supernatant liquid left after centrifugation was analyzed spectrophotometrically for myeloperoxidase levels. A comparison of exposed tissue samples readings was done with the control group in order to correlate the results with the release profile of myeloperoxidase levels and their inhibition by the prepared formulations [44].

**MTT Assay**

The cells were seeded at a cell density of 105 cells/well into 96 well plates and human skin melanoma cell line A375 and skin keratinocyte cell line HaCaT cells were incubated for 24 h at 37 °C in an atmosphere of 95% air and 5% CO₂. Cytotoxicity of cells to apigenin activity was determined by a standard spectrophotometric [3- (4, 5-dimethylthiazole-2-yl)-2,
5-diphenyl tetrazolium) MTTY assay. Then, 25 μL of apigenin, plain drug loaded gel and apigenin loaded conjugated nanotransfersomal gel, at different concentrations in PBS, was added to culture plates for 48 h. After treatment, cells were rinsed twice with PBS, and the serum-free culture medium without phenol red was replaced in all wells. Cells were then incubated for 4 h with the MTT solution (5 mg/mL). The yellow tetrazolium salt was metabolized by viable cells to purple crystals of formazan. The crystals were solubilized overnight in a mixture consisting of 20% sodium dodecyl sulfate (SDS) in HCl (0.01 M). The product was quantified spectrophotometrically by absorbance measurement at a 336 nm wavelength using a microplate reader. The cellular viability was expressed as the percentage of viable cells compared to the control group [32].

**Stability study of con-A conjugated nanotransfersomes**

To assess the ability of developed formulations, sealed bottles of the freshly prepared Con-A conjugated nanotransfersomes were placed in a stability chamber and stored at room temperature and accelerated temperature and humidity (25 ± 2 °C, 60 ± 5% RH) and (40 ± 2 °C, 75 ± 5% RH) for up to 6 months. After 6 months of storage, the samples were evaluated for structural integrity for vesicles size, zeta potential and % entrapment efficiency [32].

**Statistical analysis**

Data were presented as mean ± standard deviation and all experiments in this study were repeated at least three times. The results were statistically analyzed by analysis of variance ANOVA test; P values < .05 were considered as significant.

**Results and discussion**

**Synthesis of conjugates**

The successful synthesis of the conjugates was confirmed by IR and DSC study. The conjugation of Con-A to the nanotransfersomes was done through the crosslinking of EDC-NHS on the surface of transfersome. Con-A coupling to the nanotransfersomes was done by the carbodiimide technique. 1-Ethyl-3, 3-(dimethylaminopropyl) carbodiimide (EDC) is a water-soluble derivative of carbodiimide. N-Hydroxysuccinimide (NHS) is often used to assist the carbodiimide coupling in the presence of EDC. The reaction includes the formation of the intermediate active ester (the product of condensation of the carboxylic group and NHS) that further reacts with the amine function to yield finally the amide bond. IR studies confirmed the attachment of Con-A with nanotransfersomes, because the coupling of Con-A and transfersomes was dependent on the amide bond between NH2 group of Con-A and OH group of apigenin.

**Morphology, vesicle size and zeta potential**

TEM images showed in Figure 1(A,B) exhibit that nanotransfersomes were smaller unilamellar with a well-identified spherical vesicle confirming the vesicular characteristics indicating Con-A conjugated did not affect the vesicles structural integrity. The vesicle size of Con-A conjugated nanotransfersomal gel was found to be increased as compared to nanotransfersomes which may be due to the presence of lectin coating on the formulation. The average vesicle size of nanotransfersomes and Con-A conjugated nanotransfersomal gel was 158.5 ± 0.25 nm and 179.0 ± 0.32 nm and polydispersity index was found to be 0.199 ± 0.03 and 0.197 ± 0.07, respectively and is graphically shown in Figure 1(C,D). The zeta potential of Con-A conjugated nanotransfersomal gel was found to be increased positively as compared to nanotransfersomes, which is a property beneficial for interaction with the dermal tissues and bypass aggregation of vesicles and induced repulsive interaction between the vesicles [12]. Zeta potential of nanotransfersomes and Con-A conjugated nanotransfersomal gel was 31.06 ± 0.33 mV and 35.1 ± 0.21 mV, respectively and results are reported in Table 1 and graphically shown in Figure 1(E,F).

**X-ray diffraction study**

The X-ray diffraction, studied of pure drug apigenin, indicated an existence of sharp diffraction peak at 2θ value of 13.2°, 15.5°, 16.6°, 18° and 23°. Phospholipids showed characteristic peak 13.2° and 25.08° and Con-A displayed characteristic peak at 2.79° and 2.95°. A physical mixture of apigenin, phospholipid and Con-A in equal proportions (1:1:1) showed the appearance of newer peaks with low intensity. X-ray diffraction analysis of Con-A conjugated nanotransfersomes structure confirmed an amorphous nature which indicates the relatively low intensity observed in the nanotransfersomes. Possibly they are easy to be complexed and are graphically presented in Figure 2(A).

**FTIR Spectroscopy Study**

FTIR studies confirmed the conjugate of Con-A with apigenin loaded nanotransfersomes because coupling Con-A and nanotransfersomes depended on the amide bond between NH2 group of Con-A and OH group of apigenin. The IR spectrum of Con-A conjugated nanotransfersomal gel clearly indicated peaks (3471.1, 1735, 1690, 800–600 etc.) for amide revealing the presence of amide group in the formulation, while peaks for amide groups were absent in the IR spectrum of apigenin loaded nanotransfersomes. The FTIR spectra of apigenin showed characteristic peaks at 3550–3200 cm⁻¹ (O–H stretching), and the C–H stretching occurs above 3000 cm⁻¹ with the aliphatic C–H stretching. The very strong bands in FTIR at 3287 cm⁻¹ (C–H stretching) of aromatic group of apigenin and the C = O stretching bands are observed at in the region 1750–1735 cm⁻¹ (C = O is stretching). The observed bands at 1651, 1500, 1354 and 1245 cm⁻¹ in IR corresponds to ring modes, which are localized on the benzene part of the molecule but disappeared in the conjugated nanotransfersomal gel formulation, while the new peaks at 1559 cm⁻¹ C–C stretching and 1607 cm⁻¹ aromatic C–H bending) were appeared. Data are shown in Figure 2(B).
DSC study

Thermodynamic data from DSC thermogram is shown a sharp melting for the pure drug apigenin, Con-A conjugated nanotransfersomes, Con-A, phospholipids and carbodiimide in Figure 2(C). A sharp melting transition of the pure drug was observed at 226.98 °C and Con-A conjugated nanotransfersomes showed an endothermic peak at 174.28 °C. Con-A, phospholipids and carbodiimide showed an endothermic peak at 158.45 °C, 167.03 °C and 190.45 °C, respectively. DSC thermogram showed that the Peak intensity of heat enthalpy was reduced and slightly shifting in the melting point of drug which may be due to its solubility in the nanotransfersomes.

Table 1. Zeta potential, vesicles size and PDI of optimized formulations.

| Formulations              | Zeta potential (mV) | PDI     | Vesicles size (nm) |
|---------------------------|---------------------|---------|--------------------|
| Con-A conjugated          | 35.1 ± 0.21         | 0.197 ± 0.07 | 179.0 ± 0.32      |
| nanotransfersomal gel     |                     |         |                    |
| Marked product            | −23.05              | 0.207 ± 0.22 | 196.3 ± 0.19      |
| Nanotransfersomal gel     | 31.06 ± 0.33        | 0.199 ± 0.03 | 158.5 ± 0.25      |

Con-A conjugation efficiency and percent entrapment efficiency

The quantity of Con-A bound to the apigenin loaded nanotransfersomes was determined and the amount of Con-A was estimated by using Folin–Ciocalteu reagent. The % conjugation efficiency of Con-A conjugated nanotransfersomal gel containing apigenin was calculated as 86.8 ± 1.06%. The quantity of apigenin entrapped within con-A conjugated nanotransfersomal gel was determined by ultracentrifugation. The unentrapped apigenin in nanotransfersomes dispersion was separated by a cooling centrifuge (Remi instruments Ltd., Mumbai, India) at 13,000 rpm, for 20 min at room temperature and the supernatant was decanted. The concentration of apigenin in the supernatant was determined using UV–visible spectrophotometer at $\lambda_{\text{max}}$ 336 nm (Perkin Elmer Lambda 25 double beam UV-Visible Spectrophotometer, Singapore). Each experiment was performed in triplicate [38]. The entrapment efficiency of apigenin inside phospholipids to form Con-A conjugated nanotransfersomal was found to be 89.05 ± 0.62%. Entrapment efficiency was higher due to
the higher concentration of phospholipids and lower concentration of surfactant. This may be accredited to an increase in density of transfersomes with a decrease in surfactant concentration and also transfersomes containing Tween-80 have higher entrapment than other vesicles containing Span-80 which may be agreed by other studies [37].

Spreadability study

The spreadability value of con-A conjugated nanotransfersomal and marketed product was found to be 9.01 ± 0.32 g.cm sec⁻¹ and 7.3 ± 0.33 g.cm sec⁻¹, respectively, whereas the spreadability value of developed conventional gel formulations was found to be 6.5 ± 0.17 g.cm sec⁻¹. All the developed formulations exhibited almost similar spreadability values. This observation revealed that the incorporation of drug loaded Con-A conjugated nanotransfersomal gel into the gel base did not affect the spreadability of novel gel formulations. This might be due to the small vesicles size range of drug loaded gel giving low spreadability values indicating easy spreading on the skin.

In-vitro drug release studies

The in-vitro release studies of the drug from the apigenin loaded Concanavalin conjugated nanotransfersomal gel, pure drug suspension and marketed product were investigated in vitro over a period of 24 h using a cellophane membrane and which followed a biphasic release. The finding of our results of % cumulative drug release curve of Concanavalin conjugated nanotransfersomal gel had the lowest amount of drug release, almost 54.46 ± 0.25, observed after 24 h as compared to other pure drug suspension and marketed product dispersion was 82.80 ± 0.34 to 67.16 ± 1.22, respectively. The outcomes exhibit that the formulation represents initial burst release of the surface-adsorbed drug, was observed about 10–15% within 2 h and which followed by slow diffusion. But after 2 h release of the drug from the conjugated nanotransfersomal gel, there was towards that showed the sustained release manner. They represent the in-vitro release suggesting the burst release of drug was due to availability of the free apigenin in the outer surface of the conjugated nanotransfersomes [45]. The results of the drug release are reported in Table 2 and Figure 2(D).

Determination of drug excipients interaction study

Absorbance data of drug excipients results are presented in Table 3 for the interference of additives in the estimation of apigenin. Results observed showed that the excipients, polymer and other materials used in the preparation and
Table 2. In vitro drug release profile of Con-A conjugated nanotransfersomal gel, marketed product and pure drug suspension in skin pH 5.5.

| S.No. | Time Interval | Pure drug suspension | Conjugated nanotransfersomal gel | Marketed product |
|-------|--------------|----------------------|----------------------------------|-----------------|
| 1.    | 0            | 0                    | 0                                | 0               |
| 2.    | 2            | 12.58 ± 0.65         | 9.23 ± 1.86                     | 11.65 ± 0.26    |
| 3.    | 4            | 26.21 ± 1.12         | 20.71 ± 0.34                    | 23.11 ± 0.45    |
| 4.    | 6            | 47.3 ± 1.54          | 28.35 ± 2.07                    | 36.32 ± 0.62    |
| 5.    | 12           | 66.25 ± 1.28         | 38.04 ± 1.03                    | 51.45 ± 1.02    |
| 6.    | 24           | 82.8 ± 0.25          | 54.46 ± 0.75                    | 67.19 ± 1.34    |

Table 3. Absorbance data for the interference of additives in the estimation of apigenin.

| S. No. | Additives | Absorbance |
|--------|-----------|------------|
| 1.     | Control (5µg mL) | 0.4383   |
| 2.     | Phospholipids | 0.4290    |
| 3.     | NHS        | 0.4225    |
| 4.     | Con-A lectin | 0.4152   |
| 5.     | EDC        | 0.4260    |

Table 4. Release behaviour of apigenin from Con-A conjugated nanotransfersomal gel formulations.

| Formulation                  | First order | Higuchi | Ritger–Peppas |
|------------------------------|-------------|---------|---------------|
|                              | K          | R²      | K            | R²        | K           | R²          | n  | R²        |
| Con-A conjugated nanotransfersomal Gel | 0.086 | 0.965 | 0.406 | 0.992 | 0.653 | 0.934 | n | R²        |
| Marketed product             | 0.64       | 0.97    | 0.76 | 1.06  | 0.86  | 1.23 | K | 0.98     |
| Pure drug suspension         | 0.69       | 0.98    | 0.78 | 1.07  | 0.98  | 1.31 | K | 0.98     |

The nanosize, as well as the influence of Con-A conjugated lectins, were able to better penetrate to the entire depth of skin and also surfactant swells the stratum corneum and the intact vesicle can penetrate into and through the intact skin cells. So this permeation is required for the fabricated formulation for the efficacy of skin cancer [42].

**Histopathological studies**

The main objective of the Histopathological evaluation was to determine the targeting ability of Mice skin of the control group (group-I), UV-irradiated mice skin of control group (group-II), UV-irradiated mice skin of PDS group (Group-III) in comparison with conjugation-NTG (group-IV) and Marketed product (group-V) dermally to evaluate its topical characteristics and their effect on skin. The macroscopic effects of UV radiation on the animal’s skin were highly distinguishing for evaluation shown in (Figure 3(A)). The non-irradiated skin was free from any type of lesion formation as shown Figure 3(A)(a) while the irradiated groups developed lesion from the fourth week onwards in nearly 50% of the test animals as shown in Figure 3(A)(b). At the end of 4 weeks about 85% of animals developed photocarcinoma lesions. (extensive degradation). The cellular components like collagen, elastin, matrix proteins network were found damaged. The animals treated with free apigenin gel developed lesser skin lesions in comparison to the irradiated group as shown in Figure 3(A)(c). The animals treated with market formulation (Florida cream, Shalak Pharmaceutical Ltd, Karampura New delhi), as shown in Figure 3(A)(d), showed minimal lesion formation at the last week of the irradiation. Figure 3(A)(e), shows the skin section after 4 weeks treatment with the Con-A conjugated nanotransfersomal gel showed on discontinuation of the UV irradiations the formulation showed healing effect. The test samples showed nominal skin degradation and negligible lesion formation. So it showed that no
changes such as skin irritation or toxicity in case of edema and erythema of the treating skin.

Myeloperoxidase assay

The application of MP reduced neutrophil activation did not reduce neutrophil infiltration. But, the application of CCNTG reduced neutrophil activation as well as neutrophil infiltration also. While there were no such effects observed when pure drug suspension was applied topically to the animals. It may be due to the poor penetration ability of formulation. Although topical application of PDS, MP reduced edema, they failed to decrease the dermal neutrophil numbers, when compared with the control and standard groups. Topical treatment of PDS MP did not reduce neutrophil activation as measured by myeloperoxidase levels, while there was a significant decrease in the neutrophil activation in case of CCNTG ($P = 0.04 - 0.056$) as compared with standard ($P = .05$) and control ($P = .01$). The capability of CCNTG to reduce myeloperoxidase without disturbing neutrophil homing implies that CCNTG are selective in inhibiting the neutrophil activation and the data are shown in Figure 3(B).

Confocal laser scanning microscopy analysis

The localization of a vesicular penetration measured by CLSM study was carried out for three formulation, Con-A conjugated nanotransfersomal gel, PDS and marked product (Flondia cream, Shalak Pharmaceutical India) and graphs reported in Figure 3(C). The images of the Con-A conjugated nanotransfersomal gel have penetrated across the depth into the cell observed the internalization of vesicles well within the skin cell. Figure 3(C) Showed that the lesser extent interlunation within marketed product and a pure drug suspension. However, it is apparent that the intact con-A conjugated nanotransfersomal gel was retained in the treated skin leading to comparatively higher penetration into deeper tissues than marketed product probably due to the

| Table 5. Permeation and % drug retention data for apigenin loaded Con-A conjugated nanotransfersomal gel, marketed and pure drug suspension across abdominal goat skin. |
| Formulation | Jss ($\mu g/ ch^{-1}/h$) | P (Ch/h) | LT (hr) | Dd ($Ch^2/h$) | % drug retention after 24 h |
| Pure drug suspension | 6.01 ± 0.59 | 0.249 ± 1.42 | 3.1 | 6.12 | 0.63 ± 0.23 |
| Con-A conjugated nanotransfersomal gel | 8.42 ± 0.73 | 0.235 ± 1.56 | 2.2 | 9.6 | 0.91 ± 0.14 |
| Marked product | 7.14 ± 0.08 | 0.224 ± 1.59 | 2.7 | 7.3 | 0.72 ± 0.22 |

Jss: Transdermal flux, P: Permeability coefficient, LT: lag time, Dd: Diffusion coefficient
Table 6. Effect of apigenin, unloaded and apigenin-loaded Con-A conjugated nanotransfersomal gel on cellular viability (as % of control) of MTT assay on (a) HaCaT cells and (b) A375 cells.

| Apigenin concentration (µg/mL) | Cellular viability (% of control) |
|------------------------------|----------------------------------|
|                              | Apigenin | Marketed product | Drug loaded Con-A conjugated nanotransfersomal gel |
| (a) HaCaT cells |            |                      |                                                |
| 50                          | 110.54 ± 3.25 | 119.22 ± 2.07 | 103.54 ± 5.20 |
| 100                         | 85.11 ± 4.21 | 135.03 ± 4.24 | 126.18 ± 6.13 |
| 200                         | 72.52 ± 4.08 | 99.85 ± 8.35  | 135.24 ± 4.22 |
| 400                         | 89.41 ± 4.50 | 68.64 ± 15.09 | 48.89 ± 17.42 |
| (b) A375 cells |            |                      |                                                |
| 50                          | 107.07 ± 2.09 | 112.31 ± 5.82 | 98.12 ± 2.75 |
| 100                         | 96.41 ± 6.34 | 125.22 ± 3.87 | 120.96 ± 5.49 |
| 200                         | 82.08 ± 10.67 | 109.21 ± 8.20 | 128.04 ± 5.08 |
| 400                         | 98.42 ± 4.63 | 85.07 ± 84.32 | 40.52 ± 7.52 |

Table 7. Storage stability studies of the Con-A conjugated nanotransfersomal gel at different time interval and temperature conditions.

| Storage condition | Vesicles size (nm) | Entrapment efficiency (%) | PDI | ZP (mV) |
|-------------------|---------------------|---------------------------|-----|---------|
| Day 0             | 179.0 ± 0.32        | 89.05 ± 0.62              | 0.197 ± 0.07 | 35.1 ± 0.21 |
| 1 month at 25 °C  | 179.52 ± 0.12       | 89.15 ± 0.42              | 0.196 ± 0.15 | 35.07 ± 0.11 |
| 1 month at 40 °C  | 179.55 ± 0.28       | 89.30 ± 0.16              | 0.198 ± 0.83 | 35.13 ± 0.06 |
| 3 months at 25 °C | 179.65 ± 0.27       | 89.68 ± 0.21              | 0.198 ± 0.06 | 34.04 ± 0.86 |
| 3 months at 40 °C | 179.6 ± 0.06        | 89.69 ± 0.54              | 0.196 ± 0.69 | 34.82 ± 0.53 |
| 6 months at 25 °C | 179.7 ± 0.57        | 90.02 ± 0.11              | 0.198 ± 0.92 | 34.05 ± 0.12 |
| 6 months at 40 °C | 179.9 ± 0.74        | 90.27 ± 0.21              | 0.198 ± 0.24 | 34.69 ± 0.85 |

Cytotoxicity experiments

Cytotoxicity experiments were carried out on HaCaT cells and A375 cells lines to examine the effect on cell proliferation of apigenin as well as the marketed product and apigenin-loaded conjugated nanotransfersomal gel. Cytotoxicity assay showed that apigenin-loaded conjugated nanotransfersomal gel has significant toxicity achievements A375 compared to its low toxicity towards HaCaT as shown in Table 6(a,b) and images are shown in Figure 3(D)(a,b). The incubation with apigenin for 48 h induced a concentration-dependent inhibition of cell proliferation as determined by MTT assay. For this study, the amount of apigenin present in apigenin loaded conjugated nanotransfersomal gel was equivalent to 0.4892/5 mg. The inhibition was 18.28%, 23.34% and 32.50% at 100, 200 and 400 µg/mL of apigenin, respectively. The evaluation of unloaded and apigenin-loaded conjugated nanotransfersomal gel cytotoxicity demonstrated that, until the concentration corresponded to 200 µg/mL of apigenin, the formulations protected against apigenin cytotoxicity since no inhibition of cell proliferation was observed. Nevertheless, for the highest concentration studied, the reduction in cell viability was about 60% for drug-loaded nanoeulsion gel, due to the sum of the cytotoxic effects of both apigenin and marketed product. MTT Results showed that conjugated nanotransfersomal gel has retained the specificity of apigenin towards cancer cells without affecting the normal cells.

Stability studies

Stability of the formulated Con-A conjugated nanotransfersomes was found out of certain physicochemical properties like vesicles size, zeta potential, PDI and % entrapment efficiency and analyzed for up to 6 months. The stability studies showed that there was negligible increase in the vesicle size from 179.0 ± 0.32 nm to 179.9 ± 0.74 nm and PDI was found in slightly changes from 0.197 ± 0.07 and 0.198 ± 0.24 during the storage at room temperature and accelerated temperature and humidity. The initial % EE of the conjugated nanotransfersomes was found to be 89.05 ± 0.62%. After 6 months of storage at the same temperature conditions, it was found to be 89.05 ± 0.62% to 90.27 ± 0.21%. In contrast, there were no significant changes in the % EE during storage of formulation for 6 months. Zeta potential with slight reduction from 35.1 ± 0.21 mV to 34.69 ± 0.85 mV after 6 months storage at same temperature conditions. Thus, Con-A conjugated nanotransfersomal gel was found to be stable at room temperature and accelerated temperature and humidity for up to 6 months and results are represented in Table 7.

Conclusion

In this research study, Con-A conjugated nanotransfersomal gel was successfully synthesized by carbodiimide method with having targeted ability for better entrapment efficiency and evaluated against skin cancer by using two different cell lines. The characteristic bands of FTIR spectra certainly indicate the physico-chemical stability of the drug inside the formulation and apigenin can be attached to Con-A conjugated nanotransfersomes without altering their structural integrity. The conjugated nanotransfersomal gel was able to improve both in in-vitro and in-vivo permeation and skin deposition of apigenin compared to the marketed formulations and
retentive in the entire depth of skin for a sustainable action. These conjugates significantly improved the therapeutic efficacy and safety and possessed small vesicles sizes, and in vitro drug release study indicated the prolongation sustained release. In vivo imaging and pharmacokinetic studies revealed that conjugates had varying degrees of prolonged circulation and exhibited the lowest toxicity. Con-A conjugated nanotransfersomal gel showed reduced cytotoxicity on A375 cells as compared to HaCaT cell lines and the histological studies confirm that this approach could be used for combating the deleterious effects of ultraviolet radiations. It is concluded that developed natural drug conjugated delivery system could possibly potential effective for skin malignant melanoma.

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Disclosure statement

The authors have reported no conflict of interest.

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