Biological Forum – An International Journal **15(5a): 592-602(2023)**

ISSN No. (Print): 0975-1130
ISSN No. (Online): 2249-3239

Development and Biological Evaluation Transdermal Hydrogel for the Controlled Delivery of Curcumin against Atopic Dermatitis Management: *In-Vitro* and *In-Vivo* Studies

Umakant Sharma[1]*, Dindayal Patidar[2], Neeraj Sharma[3]
[1]Research Scholar (Ph.D.), Faculty of Medical & Paramedical Sciences, Madhyanchal Professional University, Bhopal (Madhya Pradesh), India.
[2]Professor, Faculty of Medical & Paramedical Sciences, Madhyanchal Professional University, Bhopal (Madhya Pradesh), India.
[3]Dean, Faculty of Medical & Paramedical Sciences, Madhyanchal Professional University, Bhopal (Madhya Pradesh), India.

(Corresponding author: Umakant Sharma)*
(Received: 05 April 2023; Revised: 29 April 2023; Accepted: 07 May 2023; Published: 15 May 2023)
(Published by Research Trend)

ABSTRACT: Targeting atopic dermatitis has always been a challenging task due to complex genetic phenomena and conflicting biochemical microenvironments. Drug delivery via the skin against atopic dermatitis is always been the priority based mechanism due to patient compliance and low local toxicity. By utilising lauric acid as the plasticizer, a transdermal chitosan sodium alginate biodegradable polymer mix hydrogel (CCHL) has been developed to improve the biotherapeutic properties of curcumin. Utilizing lauric acid at an optimised concentration for the physicochemical and thermostable formulation, the CCHL was prepared utilising the cold technique. When developing the biologically stable hydrogel, the optimised hydrogel was physiologically assessed using *in vitro* cell uptake, cell viability, and apoptosis assays for a complete toxicological and biologically safe margin description. The MTT assay and Apoptosis study of the produced hydrogel revealed qualitative results that were physicochemical and biologically compatible, demonstrating the considerable curcumin occupancy on human dermal fibroblast cell. By virtue of the anti-AD action, bio-pharmacokinetic parameters, histopathology and skin erythema impact, the *in vivo* DNCB based animal experiments revealed that the produced hydrogel was physiologically safe for on transdermal distribution. Overall, the current work successfully established regulated curcumin transdermal administration as a unique route to physiologically safe nanotherapy in a clinical setting. Additionally transdermal anti AD targeting comprehending natural model drug and eco-friendly delivery carrier made possibilities more contingent for the futuristic management of AD and its treatment.

Keywords: Hydrogel, Transdermal, *in-vitro* cell line, toxicology, *In-vivo*.

INTRODUCTION

After oral medication delivery, the transdermal or topical drug delivery system is the most popular and preferred method of drug administration. Numerous benefits are provided by the transdermal method of drug administration, including self-medication, low toxicity, patient compliance, avoiding dosage dumping, bypassing first pass metabolism, and painless therapy (Nagaich, 2016, Tiwari *et al*., 2012; Kothari *et al*., 2018). Since a few decades ago, cutaneous disease and other diagnostic procedures have given a great deal of importance to the transdermal medication delivery system in treating a wide range of diseases. Transdermal drug delivery including hydrogel and nanogels entails delivering medications to the epithelial layer of the skin while enhancing therapeutic effects and minimising local toxicity (Simmler *et al*., 2013; Durgavale & Rokade 2016). Many hydrogel and nanogel products are currently on the market under the label of innovative drug delivery systems, and they are used to treat a variety of diseases, including eczema, atopic dermatitis, and cancer (Ascenso *et al*., 2015; Azizoglu & Senyigit 2013).). As a result, there is a high demand for discovering and manufacturing more effective and therapeutically sound medications for transdermal delivery. Dermal malignancies hold a dignified position in the illness community in terms of infection, epidemiology, treatment, phases of diagnosis, and related infection fatality. Recent trends and statistics show that between 50 and 70 million individuals worldwide suffer from serious skin conditions such

eczema, skin cancer, and dermatitis, which contribute to 1-2 million deaths worldwide (Romero and Morilla 2013; Mustapha *et al*., 2011; Pingale *et al.,* 2023). As a result of poverty, unsanitary living circumstances, a lack of medical care, illiteracy, and other factors, contiguous skin disorders affect 4 out of 10 individuals in underdeveloped nations, making the situation much worse. The macromolecular 3D networks of the hydrogel formulations have the ability to absorb significant volumes of water or other aqueous fluids while maintaining stable physicochemical properties (Todo *et al*., 2013). The three main classifications of hydrogels—cross-linked hydrogel, covalently bonded hydrogel, and entangled hydrogel network—depend entirely on their structure and network. The pH, temperature, solvent concentration, electric field, and ionic concentration are just a few examples of the external microenvironment or stimuli that this hydrogel is extremely sensitive to. Hydrogel is a good carrier for medical applications, tissue engineering, and disease diagnosis due to all of these qualities and traits (Silverberg *et al*., 2015). The hydrogels had notable biocompatible traits and were made from a variety of polymers using both cold and heat processes. Hydrogels made of chitosan are becoming much more productive and important as a result of their reduced toxicity and improved drug loading. Additionally, chitosan has effective biocompatible properties with sustained and regulated drug release at desired areas. Its exceptional compatibility and physiochemical stability have led to its widespread use both alone and in combination with other polymers (Ozcan *et al*., 2013). We created a transdermal hydrogel using a combination of chitosan and sodium alginate polymers as well as lauric acid in a specific concentration as a plasticizer for the sustained delivery of curcumin. For adequate biocompatible and cytocompatible, assessment the transdermal biocompatible hydrogel CCHL was fabricated by previously established and published procedure using a cold technique, repeated autoclave sterilisation, and overnight storage. The developed CCHL proceed for the biocompatible studies like hemolysis and coagulation studies for investigation of the bio safe margin of transdermal drug delivery system and examining curcumin's potential and its stability placed into a 3D network of chitosan sodium alginate hydrogel CCHL. To comply with the skin microenvironment and its conditions the CCHL was further investigated for the *in-vitro* cell line studies using Human dermal fibroblast cells (HDF cell) for the proper uptake and transcytosis procedure, the plain drug curcumin and CCHL were investigated for the cell uptake, distribution, MTT and apoptosis assay for the qualitative measurement of cytotoxicity studies and biocompatible analyses on transdermal delivery. The curcumin extract is effectively held inside the hydrogel 3D network by the optimised physicochemical stable hydrogel formation, which also prevents undesirable leaking from the hydrogel formulation for the better measurement of the biocompatibility and cytocompatibilty assay (Tian *et al*., 2008). Moreover, the *in vivo* studies were carried out by using BALB/c mice utilizing 1-cholro-2, 4-dinitrobenzene (DNCB) animal model for atopic dermatitis**.** After the *in vivo* anti AD experiment the mice skin were investigated for the bio pharmacokinetic parameters, histopathology and therapeutic potential; of curcumin ageing AD when administered transdermally, here the hydrogel's surface charge is crucial for good binding to cell surface and for the skin uptake which promotes effective local cellular transport and improves bonding and internalisation at the targeted infection site. The chitosan/sodium alginate polymer network begins the sustained release of curcumin by degrading the hydrogel network at the appropriate spot upon contact with the skin's pH and its milieu, making passive transdermal drug administration more direct and efficient on a clinical platform (Azad *et al*., 2004; Hussain *et al*., 2017).

MATERIAL AND METHODS

The Chitosan and sodium alginate of medium molecular weight with75% degree of de-acetylation and acetic acid 100% ultra-pure was procured from Hi-media chemical Ltd, Mumbai, India. Pure curcumin was obtained as a benevolent gift from Taj pharmaceuticals pvt. Ltd Hyderabad. Deionized water was produced from Milli-Q Synthesis (18 MΩ, Millipore). The Human dermal fibroblast cell (HDF cell line) was procured from NCCS Pune and were maintained in Dulbecco's modified Eagles Medium (DMEM). The BALB/c mice were procured from National veterinary college Mhow, MP, India All other reagents and chemical were of analytical grade and used as received.

A. Biological Evaluations

Hemolysis. For the purpose of analysing the biological safety of the created transdermal nanoformulation, the hemolysis research was conducted on the generated CCHL formulation. For the effective start of blood compatibility research, relevant rules and ethical approval have been obtained and accepted by IAEC before beginning hemolysis investigations. About 5 ml of blood were added to 1.5 ml of acid citrate dextrose (ACD) solution and incubated for 1–2 hours at 37 °C (concentrations of the blood samples in the 100 mL ranged from 0.1 to 1 mg/ml) (Raza *et al*., 2014). To extract plasma, the incubated samples were centrifuged at 5000 rpm for 5 minutes. After that, 1 l of sodium bicarbonate solution with a concentration of 0.01% was added to the plasma. After then, the samples were scanned at 424 nm. The following equation was used to determine the plasma haemoglobin level:

$$\text{Plasma Hb} = (2A\ 294 \times 76.25)$$

Blood is prepared for the smear slide and evaluated using the CCHL push (wedge) and coverslip procedure. Using a pipette, a drop of fresh blood was applied to the slide's one end in this approach. The smear was made using a spreader slide that was 45 degrees off-perpendicular to

the blood drop to distribute the blood uniformly and prevent tailing and uneven breadth. For adequate fixing, the smear was gently air dried for 30 minutes. The fixed smear was next stained with Leishman stain (polychrome methylene blue and eosin) and floored for about 15 minutes to facilitate cell picking. After this, the smear was finally washed with water and examined using an Olympus, Japan-made fluorescent microscope with a 40x resolution lens (Jain *et al*., 2003; Godge & Ghorpade 2023).

Prothrombin Time (PT) & Activated Partial Thromboplastin Time (APTT) analysis

The freshly developed CCHL transdermal formulation's ability to induce coagulation was evaluated using the PT and APTT analytical test. To extract platelets poor plasma (PPP), fresh blood was collected in 10 ml ACD-containing tubes and centrifuged at 5000 rpm for 10 minutes at 25 °C. After combining 0.1 l of sample with 1000 l of PPP, the mixture was incubated for 25 min at 37ºC. Using a coagulation analyser reagent kit (CK Prest and Fibriprest, Diagnostica Satgo, France), the PT and APTT were evaluated after incubation (Parikh *et al*., 2018).

Cell line studies

The normal Human Dermal Fibroblast Cell (HDF) Line was purchased from NCCS Pune and kept in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 100 U/mL penicillin, and 100 g/mL streptomycin (PAA Laboratories GmbH, Austria) antibiotic solution. Cell lines were maintained at 37°C in 5% CO_2 atmosphere tissue culture flasks (75 cm^2). Cells were trypsinized with 0.25% trypsin EDTA solution (Sigma, USA) once they had reached 90% confluency. The HaCaT cells were planted in a 6 well plate (Costars, Corning Inc., NY, USA) at a density of 50,000 cells per well for the qualitative cell uptake and apoptosis analysis (Rajan *et al*., 2011).

Cell uptake studies by Confocal Laser Microscopy (CLSM)

Confocal microscopy was used to conduct advanced uptake and internalisation investigations to better understand the uptake of produced CCHL at HDF cell lines. The cells were treated with created CCHL formulation (equal to 1 g/ml) and free curcumin solution for 3 hours. Following the incubation period, the media containing the created CCHL formulation and free curcumin solution were aspirated, three times rinsed with Hanks buffered salt (HBS) solution (PAA Laboratories GmbH, Austria), and then viewed using a confocal laser microscope (CLSM) (Olympus) (Thomsen, 2014; Kundu *et al*., 2022).

MTT Assay

The CCHL formulation's cell cytotoxicity on HDF cells was assessed using the MTT test. In a nutshell, the cell line was shown in 96-well plates, cultured with media containing free curcumin solution, and generated CCHL formulation (Equivalent concentration of 0.1 to 1 g/mL, negative control (cells treated with blank media), and positive control (Triton X-100). Following a 24-hour incubation period, the samples' medium was aspirated, and cells underwent three HBSS washes. Following that, each well received 150 L of MTT solution (500 g/mL in PBS), which was then re-incubated for 4 hours. After 4 hours, the formazan crystals were carefully aspirated from the MTT solution and dissolved in 200 L of DMSO. Then, an ELISA plate reader (BioTek, USA) was used to measure the optical density (OD) of the resulting solution at 570 nm (Kanwar and De 2011).

Apoptosis Studies

The capacity of the proposed CCHL formulation and free curcumin solution to induce apoptosis in the HDF cell line served as a measure of their cytotoxic activity. The phosphatidyl serine exposure on the plasma membrane outer layer and its interaction with Annexin V are the foundation of the Annexin V apoptosis test. on a nutshell, the cells were placed on a 6-well cell culture dish and maintained there for an overnight period at 37°C and 5% CO_2. Media containing free drug and drug-loaded NPs (equal to 10 g/mL) was then aspirated, replenished, and incubated for 6 hours. After the cells had been incubated for 6 hours, the medium was removed, and the cells were then treated with 6-carboxyfluorescein diacetate (6-CDFA) and Annexin V Cy3.18 (AnnCy3) (Annexin V Cy3TM Apoptosis Detection kit, Sigma, USA) (Malakar *et al*., 2012). Then, HaCaT cells were seen using CLSM with 6-CDFA and AnnCy3 in the green and red channels, respectively. Apoptosis index was also calculated as the fluorescence intensity ratio of the red (measure of apoptosis) and green (a measure of viability) channels. The U.S. National Institutes of Health, Bethesda, Maryland, USA, used Image J software to measure the fluorescence intensity in the photographs (Kanchongkittiphon *et al*., 2015).

***In Vivo* studies**

Animal Studies

The BALB/c male mice of weight 25-30g were used for the *in-vivo* anti atopic dermatitis studies. Animals were procured from the Veterinary College, Mhow (MP) India. Animal experiment was carried out in Madhyanchal Professional University Bhopal (MP) India, after due permission of institutional animal ethics committee reference no. 1698/PO/Re/S/13/CPCSEA, which is registered with the committee for Control and Supervision of Experiments on Animal (CPCSEA), government of India (McPherson, 2016). The mice were maintained under the standard laboratory conditions at 27+ 2 °C with relative humidity of about 50-70% and at optimum photoperiod (12 h darkness/ 12 h light cycle) for carrying out *in-vivo* studies. The mice were normally fed with normal diet chew procured from Hindustan Lever Ltd., Mumbai, India and water was provided ad libitum and bedding was changed twice a week to safeguard the optimum hygienic environments and good comfortable conditions to animal (Park, 2014).

***In vivo* Anti AD experimental studies**

BALB/c mice were used for the *in vivo* experiments. The institutional ethics committee examined and approved

the study. With the use of 1-cholro-2, 4-dinitrobenzene (DNCB), atopic dermatitis was developed. The animals were separated into four groups (I, II, III, and IV), each of which had four of the creatures. Animals in groups II, III, and IV received 200ml of 0.5% DNCB in acetone (3:1) for 3 days to be administered to the shaved region. On Days 14, 17, 20, 23, 26, and 29, mice received 200 cc of 1% DNCB as a treatment (Rerknimitr *et al*., 2017). A prepared dosage form (CCHL) and plain curcumin solution were administered to the backs of the animals in groups III and IV, respectively, 30 minutes after the administration of 1-cholro-2, 4-dinitrobenzene. Animals were sacrificed to remove afflicted skin patches following the whole course of therapy to assess changes in erythema score, scratching score, and haematological parameters. A complete blood count test was used to assess changes in blood parameters. Whole blood samples were obtained for this test and placed in EDTA tubes. The following group were assigned for the *in vivo* treatment experiment (Siddique *et al*., 2016).

G1: Control group
G2: The disease-induced group's (negative control).
G3: Treatment Group (plain curcumin solution)
G4: Treatment Group 2 (CCHL)

Biochemical Parameter Estimation

The blood samples were collected from mice in sterilized centrifuged tubes for the biochemical investigation of enzyme levels *viz*., Catalase (CAT), Superoxide dismutase (SOD), Lipid peroxidase (LP) and Glutathione (GSH) using the ELISA enzyme detection Kit. The collected blood was then centrifuged at 3000 rpm for about 10 min to obtain clear serum (Toussaint *et al*., 2014).

Histological Assessment

The skin sample from each mouse was removed after dissection and rinses with normal saline (09% w/v) blotted on Whattman's filter paper, weighed and homogenised with PBS solution (pH 7) and fixed in 10% formalin for 48 h and embedded in paraffin followed by carefully sectioned and microscopically examined stained by haematoxylin and eosin dye to screen the pathological changes by using Fluorescent microscope (Olympus, Japan) under 10x, 45x and 100x objective lens (Williams and Gallo 2017).

Statistical Analysis

The values were expressed as mean $\pm$ SD. The statistical analysis was performed by employing one way ANOVA followed by a DUNNET'S T- test. The obtained value will consider statistically significant, if p value is less than 0.01 (P value < 0.01)(Zaniboni *et al*., 2016).

RESULTS AND DISCUSSION

A. Hemolysis Assay

Patients who received *in vitro* infusions of any drug or API on the clinical platform had changes in the shape, fluidity, and ionic balance of their erythrocytes. Red blood cells' capacity to carry oxygen is decreased as a result, and this causes an inadequate blood supply to the heart, which can cause cardiotoxicity. In order to protect the direct exposure of drug in blood circulation and efficiently transport the drug load to the infected (AD) environment, we hypothesised to create a transdermal CCHL hydrogel with sustained drug release of curcumin. When compared to positive control, the CCHL blood compatibility effect at various concentrations showed a striking reduction in hemolysis effects, as shown in Fig. 1 a-h. Significant analysis reveals that the hemolysis impact only manifests in the positive control vial, whereas neither the negative control nor the formulation vials showed any RBC lysis. In contrast to the positive control, which displays opaque red-colored plasma suggesting the lysis of RBCs and the leaching out of haem and other components, the clear plasma at concentrations of 0.2 to 0.8 reveals no RBC lysis (Fig. 1 i). RBC hemolysis is barely detectable when CCHL concentrations are between 0.2 and 0.6 mg/ml, while a minimal increase in hemolysis is observed when CCHL concentrations are between 0.8 and 1.0 mg/ml and higher. The CCHL hemolytic ratio displays less than 4%. According to ISO/TR 7406's ethical course of action, which permits a maximum of 5% hemolysis, this is regarded as safe in clinical settings, indicating that the CCHL satisfies essential clinical safety requirements. We saw blood samples using a light microscope with Leishman's reagent staining or bright field pictures in order to further assess the hemolytic effect of CCHL on erythrocyte shape. RBC lysis and coagulation for the positive control group, however no detectable coagulation or rupture of RBCs was seen in any concentration of CCHL-treated blood. The hemolysis results demonstrate that CCHL has little toxicity to erythrocyte lysis and is effective for treating atopic dermatitis. As the minor nanoformulation penetrates the blood circulation after demonstrating its potency in a targeted and desired region on extended exposure and retention, the hemolysis findings effectively verified the permeation tests and penetrating potention of CCHL on transdermal administration. The *in vitro* Hemolysis investigation successfully supported the hemolytic ratio's justification of the synthetic CCHL biological safe margin and showed that, with transdermal treatment, the formulation (in minute traces) also enters the blood circulation.

B. PT & APTT Analysis

The developed CCHL formulation must first undergo PT and APTT screening before being used in nanomedicine. The extrinsic process of blood coagulation is demonstrated by the PT evaluation, while the intrinsic pathway is revealed by the APTT. Both the blood compatibility parameter and the biosafety margin of the nanoformulation verify the possible CCHL treated blood coagulation phenomena. When compared to the negative control, the free curcumin and CCHL formulation's coagulation times in the PT and APTT evaluations were 12.3 & 13.1 seconds and 29.4 & 30.7 seconds, respectively, demonstrating the typical safe margin of the nanoformulation. Due to the cationic

character of CCHL and the fact that blood RBCs have a negative charge on their membranes, the investigations showed that the CCHL nanoformulation does not disturb either the intrinsic or extrinsic route of blood coagulation (Fig. 1j). Therefore, the biosafety margin of BCNL is shown to be insignificant.

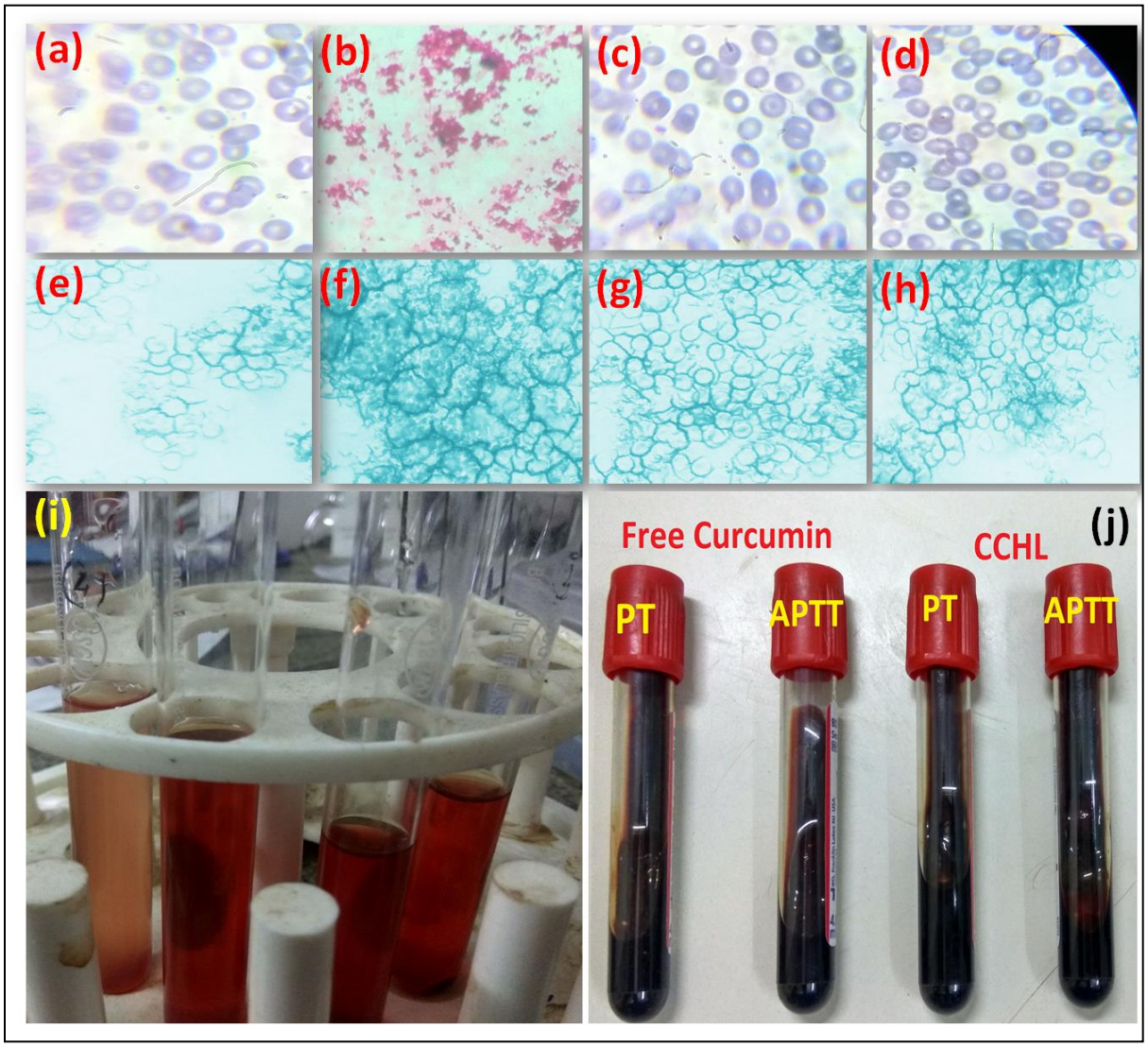

Fig. 1. Illustration of Hemolysis studies of different groups depicting microscopic visualization with phase contrast image of blood treated groups, images (a & e) control group, (b& f) negative group, (c& g) free curcumin and (d& h) CCHL, whereas image (i) showing different vials of hemolysis process holding blood with different groups and image (j) showing PT and APTT profile of curcumin and CCHL post coagulation process, (mean + SD, n=3), *p<0.05 and *p<0.01 compared to the normal control.

C. Cell uptake assay

The cell uptake assay along with CLSM images of HaCaT cells treated with free curcumin and developed CCHL (1 g/ml, 4 h) in the qualitative uptake investigation were measured. As can be seen from the image, CLSM noticed much more fluorescence in cells treated with CCHL than in cells treated with free curcumin. This is likely due to the nanoformulation's successful cellular absorption or internalisation. Additionally, a horizontal series line analysis was assessed, which demonstrated increased green intensity and was discovered to overlap with white line deviation (white line deviation is caused by cell structures) in the case of CCHL. While the green fluorescence of free curcumin overlaps as well, it is substantially less intense than the green fluorescence of CCHL (Fig. 2). The aforementioned finding indicates that the fluorescence was exclusively a result of internalised free medication and not from free curcumin that had been adsorbed on the cells' surfaces (Fig. 2a). Thus, it can be quickly determined that the internalisation mechanism for the nanoformulation absorption process might be size dependant. However, different cell lines display various endocytosis mechanisms, and numerous uptake routes may be active at once.

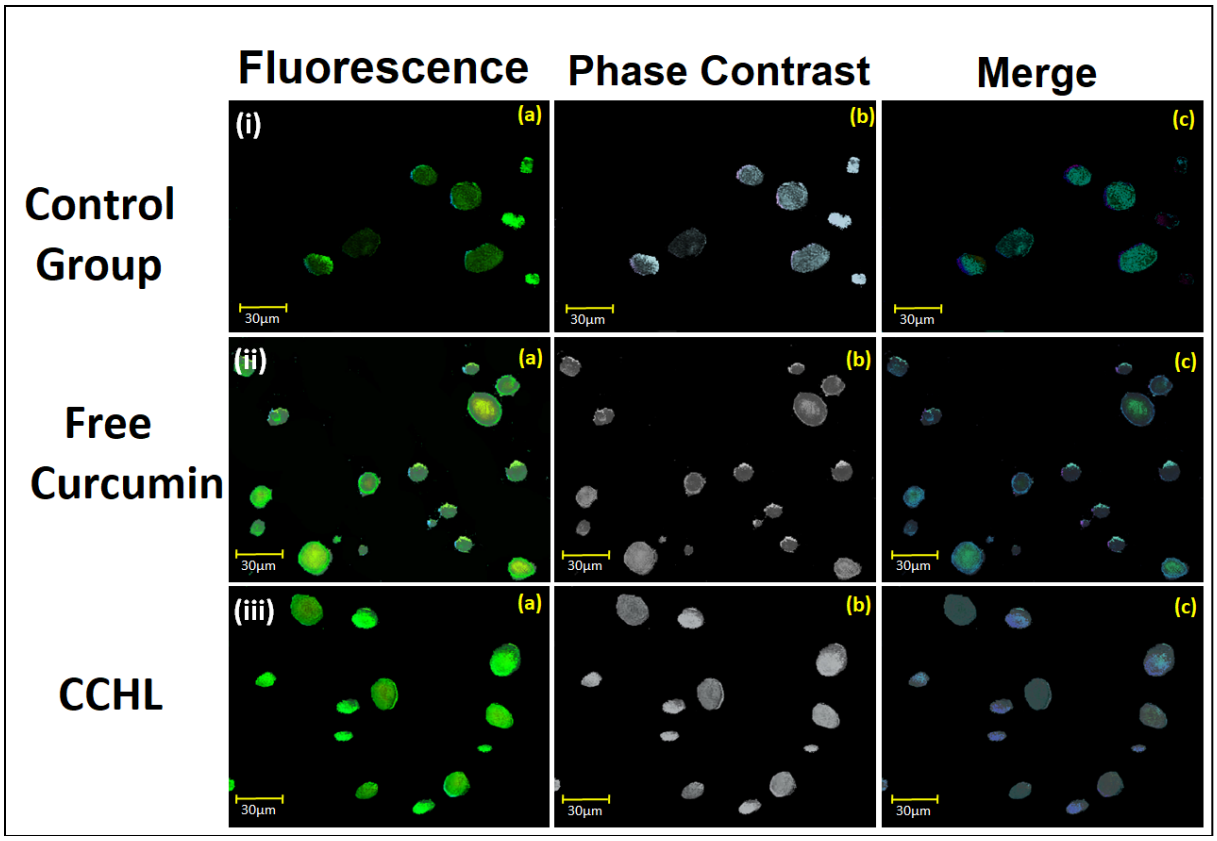

Fig. 2. Cell uptake profile by confocal microscopy (i, a-c) control group, (ii, a-c) free curcumin group and (iii, a-c) CCHL group on HDF cell line post incubation for 12 h, (mean + SD, n=3), *p<0.05 and *p<0.01compared to the untreated cell.

D. MTT assay

The MTT cytotoxicity result demonstrates that plain curcumin solution and CCHL are cytocompatible at all concentration (Fig. 3b). The cytotoxicity result showed that the CCHL nanoformulation is slightly more toxic to HDF cell compare to free curcumin exhibiting enhanced cell cytotoxicity effects of CCHL by HDF cell and found statistically significant when analysed by the student's T test. The developed CCHL and free curcumin shows noteworthy cell toxicity towards the human HDF cell line due to the free radical scavenger property curcumin, which exhibit selective toxicity towards cancer cell rather than normal cell.

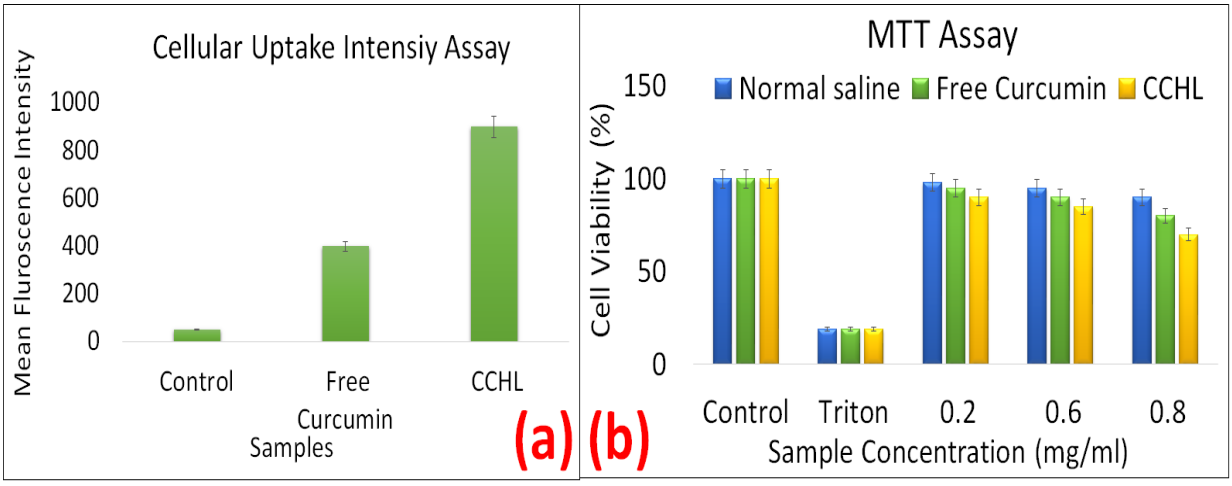

Fig. 3. Elaboration of cellular uptake intensity analysis (image a) of different group on human HDF cell line post incubation for 12 h, whereas image b elaborating cell viability assay (MTT assay) by different group and human HDF cell line incubated for 12 h respectively (mean + SD, n=3), *p<0.05 and *p<0.01 compared to the normal control.

The blank developed CCHL, composed of outer chitosan boundary and 3d network entrapping curcumin showing enhanced toxicity and had an impact that was similar to that of direct systemic exposure. Biodegradable chitosan CCHL and the anti-AD compound curcumin work together to lessen the toxicity on normal HDF cells. The CCHL is a perfect transdermal nanocarrier against atopic dermatitis with little toxicity towards normal cells, according to the MTT experiment, which also showed that it preserved the potential and power of the curcumin towards AD cells while avoiding toxicity to the normal.

E. Apoptosis Assay

The results of apoptosis suggest that both CCHL and free curcumin display competent apoptotic capability at greater concentrations but developed CCHL showed enhanced apoptosis compared to free curcumin (Fig. 4). The anti-AD potential rich curcumin displays an inherent apoptotic impact through a variety of phenomena. The effects of curcumin on apoptosis are both intrinsic (mitochondrial) and extrinsic (Death receptor). The primary cause of innate apoptosis is curcumin, which causes it by acting during the G2 phase and demonstrating the mitochondrial apoptosis mechanism. By activating a cell surface receptor, curcumin functions as a death activator in the extrinsic apoptosis pathway phase. Initiating the cell surface receptor, activation of caspase-8 and initiation of caspase cascade take place by curcumin. By allowing CCHL to enter through the stratum corneum or hair follicle channels by endocytosis and interacting with the negatively charged cell membrane of atopic dermatitis, the cationic charge of CCHL also facilitates notable apoptosis. As a result of the charge interaction or ionic attachment, the chitosan core may be degraded effectively, releasing an anti-AD curcumin at the desired location without disrupting the normal cells, demonstrating the biosafety and cytocompatibility of the CCHL hydrogel system.

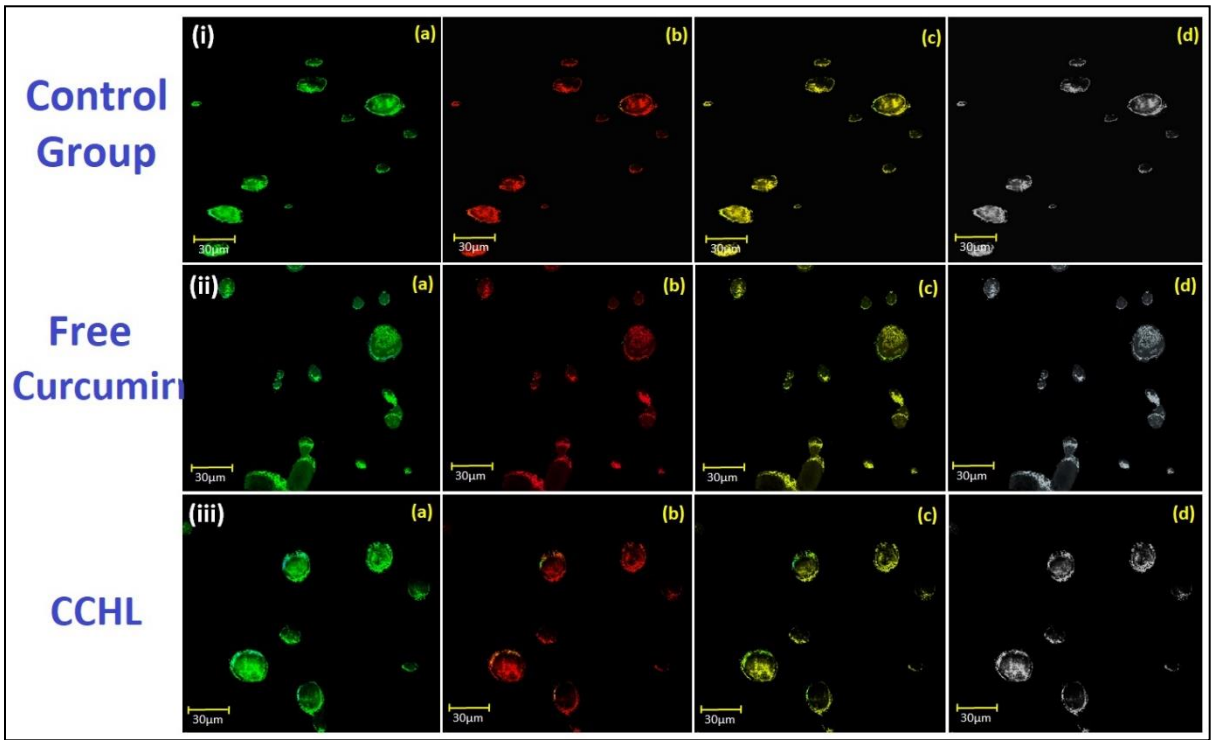

Fig. 4. Illustrating apoptosis assay by different group on human HDF cell line images (i) control (10 µg/ml; 6h incubation), (ii) free curcumin (equivalent to 10 µg/ml and incubated for 6 h) (iii) CCHL (equivalent to 10 µg/ml and incubated for 6 h) against HDF cells (III-V, a) Green channel depicts the fluorescence from carboxyfluoresce (cell viability marker dye); (III-V, b) Red channel depicts fluorescence from Annexin Cy3.18 conjugate (cell apoptosis marker dye); (III-V, c) Overlay image of figure (a) and figure (b); whereas, (III-V, d) Depicts the differential contrast image (DIC) of representative cells, (mean + SD, n=3), *p<0.05 and *p<0.01 compared to the untreated cell.

F. In vivo studies

Using BALB/c mice, the produced ultra-deformable vesicles loaded hydrogel containing curcumin was evaluated for its *in vivo* pharmacodynamic effects on atopic dermatitis. According to the findings *of in vivo* investigations, formed hydrogel was effective in treating both erythema and scratching scores in animals that had been given atopic dermatitis. Scratching score and erythema score findings are shown in Fig. 5 (a & b), respectively. It is evident that, when compared to the other groups, the control and negative group showed the greatest decrease in the scratching and erythema scores. The epidermis is the top layer of healthy skin, while the dermis is found underneath. During atopic dermatitis,

these layers are destroyed when 1-cholro-2, 4-dinitrobenzene is applied to the skin's surface. When mice were exposed to 1-cholro-2, 4-dinitrobenzene, it seemed as though their skin layers were ravaged. The damaged skin layers were healed and mended after application of both designed hydrogel and ordinary plain drug-loaded hydrogel. The damaged skin layers were healed and mended after application of both group G3 (free curcumin) and G4 (CCHL) hydrogel group.

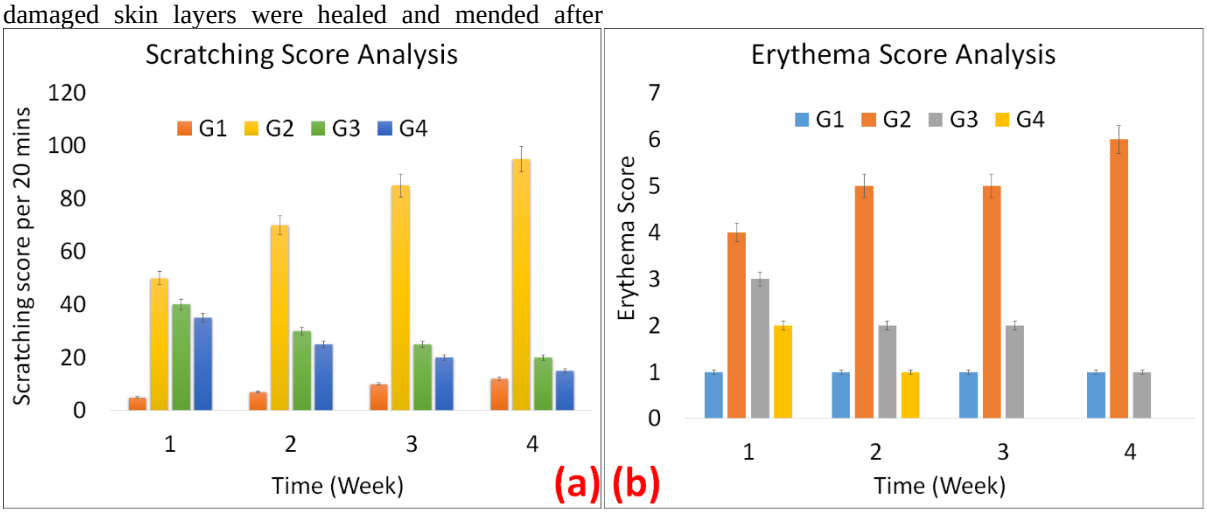

Fig. 5. Illustration of *in vivo* scratching score analysis (image a) and mean erythema score analysis (image b) by different animal treated groups in 4 week *in vivo* animal studies (mean + SD, n=3), *p<0.05 and *p<0.01 compared to the untreated group.

Biochemical Oxidative Stress Enzymes Estimation

On the skin tissue and blood of treated animals, the levels of the oxidative stress enzymes CAT (Catalyse), GSH (Glutathione), LP (Lipid peroxidase), and SOD (Superoxide dismutase) were examined. The negative group (G2) revealed significant variations in all of the enzyme levels upon measurement of biochemical oxidative stress enzymes as a result of prolonged exposure to carcinogens. In a 4-week *in-vivo* investigation, the GSH (12.9%), CAT (15%), and SOD (14.9%) showed discernible level declines (P<0.05), whilst the LP level indicated 63% (P>0.01) increased peroxidation activity in comparison to the normal control (G1). Due to a powerful anti-metabolic pathway inhibitory impact in blood and tissue, the G3 group free curcumin treated animal showed a noteworthy drop in LP level of 20% while an increase in GSH (21.2%), CAT (20.1%) and SOD (19.4%) levels was seen in comparison to the normal (G1) and toxic group (G2). When compared to the normal and control groups, the G4 CCHL treated mice showed a significant increase in GSH (34%), CAT (37%) and SOD (31.8%) levels, while an enhanced decline of 11% (P<0.01) in LP level was seen (Fig. 6 a &b). These results demonstrate the remarkable anti-AD effect of curcumin intercellularly.

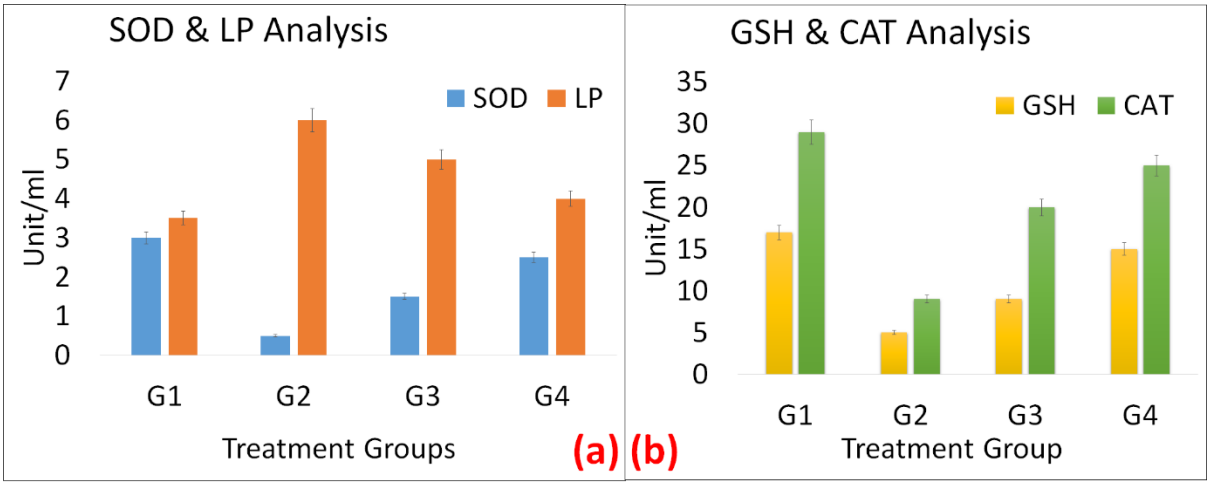

Fig. 6. Elaboration of Biochemical Oxidative Stress enzymes estimation of CAT (Catalyse), GSH (Glutathione), LP (Lipid peroxidase), and SOD (Superoxide dismutase) by different treatment groups in 4 weeks *in vivo* animal skin extraction studies, (mean + SD, n=3), *p<0.05 and *p<0.01 compared to the untreated group.

The Anti-AD effect of curcumin loaded CCHL, which demonstrated considerable apoptosis in treated mice and resulted in a notable reduction of AD cell *in-vivo*, is responsible for the increase in GSH, CAT, and SOD and drop in LP level in G5. Additionally, CCHL gel demonstrated greater diffusion and curcumin discharge at the targeted site (AD) when compared to bare curcumin owing to better transcytosis. Therefore, compared to bare curcumin and commercial other group, the combination of biodegradable chitosan and potent Anti-AD curcumin facilitates stronger anti-atopic dermatitis effect action on transdermal administration.

G. Histopathology assay

In comparison to other treatment groups, the histology profile obtained from the treated mice showed strong anti-AD potential for CCHL (Fig. 7 (a-d). In the atopic dermatitis 4 week animal model, the animal treated with carcinogen (G2), the histology results showed a wide spectrum of disruption in the squamous layer of skin with keratin outflow. There was obvious evidence of the organised lipid bilayer loosening along with severe cellular necrosis. Animals treated with free curcumin (G3) showed signs of epithelial layer regeneration and resurrected keratin pearls, as well as healing of the squamous layer and healthy subcutaneous tissue presence. In animals treated CCHL (G4), the stratum corneum was remarkably restored, showing a well-regenerated keratin layer and globules. The area of subcutaneous tissue was better restored than the G2 and G3 therapy animals and is essentially identical to the normal control. The epithelial layer had a typical appearance, was well-aligned, and seemed to be the same as in G1 animals. The considerable histology results of G4 once again demonstrated chitosan loaded curcumin hydrogel higher anti-AD potential compared to free curcumin solution. In comparison free curcumin solution, the penetration was meagre and early adsorption over skin deprive the therapeutic potential compared to CCHL into the stratum corneum. The effectiveness of CCHL skin absorption is two times greater than that of free curcumin, demonstrating the critical role of therapeutic anti AD that play crucial role in transdermal administration. The benefits created by *in-vitro* cell lines investigations were subsequently corroborated by the histology research, which made sure that developed CCHL existed and was distributed better in the targeted area compared offering up new avenues for the development of targeted drug delivery and against atopic dermatitis (AD).

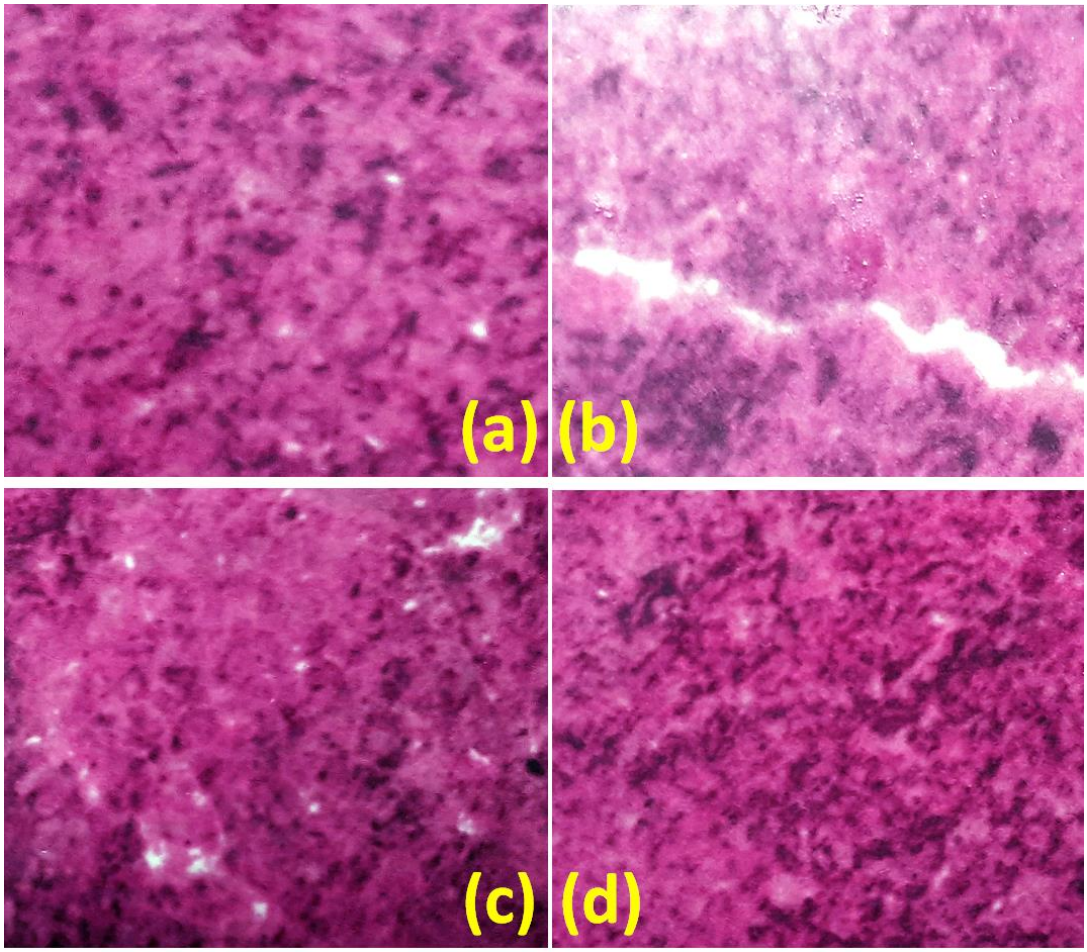

Fig. 7. Elaboration of histopathology analysis of various treatment group (a) G1, (b) G2, (c) G3, & (d) G4 in 4 week *in-vivo* animal study, (mean + SD, n=3), *p<0.05 and *p<0.01 compared to the normal control.

CONCLUSION

The results demonstrate the qualitative penetrating potential of biodegradable cationic chitosan loaded curcumin hydrogel offering pH triggered and charge rebound innate mechanism enabling microenvironment dependent anti AD therapeutic effect and improving curcumin release with decreased local cellular toxicity. As shown by *in vitro* cell line studies and hematological studies therapeutic transdermal targeting evaluation, ionic interaction and EPR dependent absorption of CCHL enable onsite targeting and subsequent toxicity on HDF cell line, lowering atopic dermatitis infection burden on skin area. Discovering a unique transdermal biodegradable therapy with harmless constituent's usage the advanced *in-vivo* evaluation showed increased diffusion of CCHL and optimal retention at the targeted dermatitis location with negligible cellular toxicity at low dose of curcumin. The fabricated hydrogel demonstrated the most extreme Anti-AD specificity with lowered dose and duration, improving therapeutic efficacy for atopic dermatitis patients with increased skin rehabilitations rate, and paving the way for simple and effective clinical transdermal nanotherapy against atopic dermatitis.

FUTURE SCOPE

The current research needs more attention on the light of clinical studies to enhance the dose duration and detailed local side effects. Additionally, comprehensive optimizations parameters including process and formulation elements needs more attention to attain optimum dose size on administration along with additional cytotoxicity and bio-toxicity studies must be taken in account for the extreme biological safe margin for the safest transdermal drug delivery system attainment not only for AD but also wide variety of dermal diseases.

Acknowledgement. The authors are thankful to Prof. (Dr.) Sailesh Kumar Ghatuary, Director, Shri RLT Institute of Pharmaceutical Science and Technology, Ekdil-Etawah, Uttar Pradesh, India, for his assistance and providing the valuable suggestions.

Conflict of Interest: The author declares no conflict of interest.

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How to cite this article: Umakant Sharma, Dindayal Patidar and Neeraj Sharma (2023). Development and Biological Evaluation Transdermal Hydrogel for the Controlled Delivery of Curcumin against Atopic Dermatitis Management: *In-Vitro* and *In-Vivo* Studies. *Biological Forum – An International Journal, 15*(5a): 592-602.