Original Article

Formulation Development And Pharmacological Evaluation Of Topical Hyalurosomes Nanogel Of Calotropis Gigantea For The Treatment Of Vitiligo

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Abstract

Objective: The objective of the paper is preparation and pharmacological evaluation of topical hyalurosomes nanogel of *Calotropis Gigantea* for the treatment of Vitiligo.

Material and methods: Fresh milky white latex of *Calotropis Gigantea* was collected by cutting of stem twigs and identified by FT-IR for the presence of active phytoconstituents. Hyalurosomes as nanogel formulation was prepared by solvent evaporation method. The nanogel for anti-vitiligo activity was evaluated and performed by utilising cell viability, melanin content, tyrosinase activity assay and Fontana-Masson silver staining on B16F10 cell line.

Result: The FT-IR analysis has shown the broad peak at 3253 cm^{-1} to OH-str. frequencies of uscharin, calotropin, lubeol, and b-amyrin. The Petroleum ether latex of *Calotropis Gigantea* contain i.e., lupeol, beta-amyrin, alpha calotropeol, and beta-calotropeol were utilized as drug for the topical nanogel delivery. The Cell viability assay stated that CGHGF showed 68.71±2.76% cytotoxicity, as compared to CG-Carbopol Gel (52.10±9.63%) at 50µM concentration. The MTT assay stated the CG-Carbopol gel show 56.11±11.55% and CGHGF has shown 82.95±11.61% cytotoxicity at 50 µM concentration. Melanin and tyrosinase activity stated that petroleum latex of *Calotropis Gigantea* solution showed an increase in melanin content by 1.20 fold upon first treatment ($p<0.05$) and 1.20 fold upon second treatment ($p<0.05$). CGHGF is showed a 2.25 fold increase in melanin with first treatment ($p<0.0001$) and a 2.89 fold increase with second treatment ($p<0.0001$). Fontana-Masson silver staining is performed to visualize melanin pigment in cells exposed to *Petroleum latex of Calotropis Gigantea* CGHGF formulations. Compared to the control, the amount of melanin granules was significantly increased and stained in petroleum latex of *Calotropis Gigantea* based nanogel.

Discussion: Unique deformable nature of hyalurosomes and CG Carbopol gel also assists in skin penetration enhancement of latex containing active constituents. Cell uptake studies confirmed higher penetration of encapsulated moiety through prepared CGHGF.

Keywords: Hyalurosomes, *Calotropis Gigantea,* HPLC, B16F10 cell line, cell uptake

INTRODUCTION

Vitiligo (leukoderma) is recognized as a common skin pigmentation disorder stemming from progressive selective destruction of epidermal melanocytes operating as pigmentation cells. Vitiligo is an acquired idiopathic, dermatological disorder characterized by well circumscribed milky white macules devoid of identifiable melanocytes.[1] These asymptomatic white macules can be psychologically extremely damaging, even leading to attempted suicide in some cases. It affects approximately 1% of the world's population and approximately 3-4% of the Indian population. The most common sites of involvement are the face (24.5%), neck (18.8%), and scalp (11.2%). The vitiligo results in obvious flat white lesions in normally pigmented skin, which commonly appear on face, arms, hands, feet, and lips. Patches may be progressive and arise at any age.[2]

Exposure to environmental stressors, such as ultraviolet (UV) radiation and numerous chemicals, usually affects epidermal cells, including melanocytes, which leads to an increase in the production of reactive oxygen species (ROS). Vitiligo patients have intrinsic defects in their melanocytes, which diminishes the cell ability to respond well to cellular stressors. Therefore, increased concentrations of epidermal H_2O_2 and decreased concentrations of catalase, which protect cells from oxidative destruction, have been found. The rationale of vitiligo treatment is to control immunoreactions by suppressing oxidative stress and restoring the normal skin colour in the affected area through formation of healthy melanocytes instead of damaged ones. [3]

To date, no curative therapy is available for vitiligo. In many years, vitiligo therapy has depended on systemic therapy, topical agents, phototherapy, and surgical techniques, which all aim to reduce disease progression and stimulate skin re-pigmentation. The chronic nature of the disease and long-term therapy with the lack of uniform treatment are very demoralizing for vitiligo patients. The natural treatment approaches for management of vitiligo have been concerned such as using hypericin, khellin, and berberine are considered as effective therapeutic tools for the treatment of vitiligo.[4]
Recently, Nano-dermatology science applies nanotechnology approaches in the field of dermatology and have involved sunscreens and maintenance of skin health as well as providing a tool for the diagnosis and management of skin disease and transport bioactive compounds at effective concentrations over a predetermined period. Such tactic has been directed toward enhancement of the activity problems of many biologically active compounds via improving drug solubility, skin deposition, skin permeability as well as minimize toxicity.[5]
Sodium hyaluronate (hyaluronan) is the sodium salt form of hyaluronic acid that present in human organs as part of numerous connective tissues, lungs, synovial fluid, and muscle tissues. It is considered to be a dual functioning component that has been used as a viscosity enhancer in skin care products and to improve dehydrated skin by replenishing the hyaluronic acid content.[6] Hyalurosomes are a modified nanovesicles that possess the intrinsic characteristics of phospholipid nanovesicles potentiated with hyaluronan penetration enhancer and gelling capabilities. Consequently, hyalurosomes combine the privileges of both elastic features of deformable liposomes and the stability of gel-core vesicles. Hyalurosomes would provide many benefits in local skin delivery owing to hyaluronan such as; longer residence time at the application site, penetration enhancing ability, hence facilitating skin permeation and drug deposition.[7]
Therefore, loading of drug in hyalurosomes are expected to yield an outstanding outcome by enhancing the low skin permeability of *Calotropis Gigantea* latex providing promising antioxidant and anti-inflammatory effects. Consequently, the current study is the first work to represent topical *Calotropis Gigantea* latex-hyalurosomes nanogel as a targeted nano-therapy to enhance the skin permeability and deposition of drug for treatment of vitiligo.[8]
The natural product obtained from the plant sources pay attention towards the management and treatment strategies for Vitiligo patients. The main approaches of the research work are to focus on the herbal based nano-formulation (hyalurosomes) Petroleum latex of *Calotropis Gigantea* to enhance therapeutic activity and skin permeation and evaluated them for anti-vitiligo activity. The topical route may improve the bioavailability and efficacy of the treatment of Vitiligo as well as minimize toxicity.

MATERIALS AND METHODS

Lipoid® S100 (l-α-phosphatidylcholine) was obtained as gift sample from Lipoid AG (Ludwigshafen, Germany). Sodium hyaluronate (hyaluronan) was Purchased from Euromedex, (France). The Petroleum ether Latex of *Calotropis Gigantea* were used as drug for encapsulation in polymeric nanocarrier. Hydroquinone and carbopol-940 were purchased from Sigma-Aldrich, New Delhi. Sodium deoxy cholate (SDC), bovine serum albumin, acridine orange (AO), nuclear fast red and cellulose dialysis tubing with 12000 Da molecular weight cut off was purchased from Hi-Media, Mumbai, India. Chloroform, methanol and acetonitrile were obtained from Merck Pvt Ltd. Mumbai, India. All other chemicals and reagents used were of analytical grade.

Extraction and Identification of *Calotropis Gigantea* plant

Fresh milky white latex of *Calotropis Gigantea* was collected by cutting of the stem twigs. Moist Lax was dried under open air during the day.[9] The dried Lax was divided in two parts; the first sample was evaluated for its solubility in various solvents such as hexane, ethyl ether, chloroform, ethyl acetate, butanol, methanol, acetone, acetonitrile, and water. The maximum solubility was observed in petroleum ether and methanol. The presence of active constituents was defined by the IR analysis.

Preparation and characterization of Topical hyalurosomes nanogel (CGHGF)

Solvent evaporation method was used for the preparation of hyalurosomes nanogel with some modification.[10] Briefly L-α-phosphatidylcholine (5% w/v) and sodium deoxy cholate (SDC; 3.25%w/w) was dissolved in 2 ml of absolute ethanol. Then, the resulting ethanolic solution was injected dropwise through a 23-G syringe into a 10-ml hydrating medium solution containing hyaluronan (2.5%w/v) and petroleum ether latex of *Calotropis Gigantea* as drug (0.25%w/w, 5 mg) in water: ethanol (80:20) under constant magnetic stirring at 1500 rpm and room temperature for 90 min. Hyaluronan was used as a self-gelling agent at a concentration of 2.5%(w/v). The placebo- hyalurosomes (CGPHGF) was prepared by same procedure without using the latex (drug).

Preparation of conventional Carbopol gel formulation loaded with Petroleum ether latex of *Calotropis Gigantea* (CG-Carbopol Gel)

A petroleum ether latex of *Calotropis Gigantea*-loaded conventional Carbopol hydrogel was prepared as a control gel for comparison with hyalurosomes. [11] Gel was prepared by dispersing 5 mg of petroleum ether latex of *Calotropis Gigantea* (0.25% w/v) in 20 ml of distilled water. One gram of carbopol-940 was added portion-wise under magnetic stirring until a gel was obtained at room temperature.

PHARMACOLOGICAL EVALUATION

Evaluation studies on B16F10 cell line

Melanocyte cells (B16F10) used in the study were procured from the National Center for Cell Science, Pune and were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% heat inactivated fetal bovine serum, 1.5g/L $NaHCO_3$, 2mM L-glutamine, 10,000 units penicillin, 10µg/mL streptomycin, and 25μg/mL amphotericin B, incubated at 37°C with 5% CO_2 in a humidified atmosphere. [12]

Cell Viability Assay

Cells were seeded in 96-well plates at a density of 1×10^4 cells per well and incubated for 24h. Initially cells were treated with *Petroleum latex of Calotropis Gigantea* as drug (0.25%w/w, 5 mg) solutions at 10 to 250µM equivalent drug concentrations to obtain 50% cell growth inhibitory concentration (IC_{50}) for the respective drugs. After 24h incubation period, fresh DMEM containing 500µg/ml of 3-(4,5-Dimethylthiazol-2-yl)]-2,5-diphenyltetrazolium bromide (MTT) was added to replace the formulations and incubated for 3h. [13]

MTT solution was aspirated and dimethyl sulphoxide (DMSO) was added to dissolve the formazan crystals. Absorbance was measured at 570 nm using microplate reader (Spectramax, Molecular Devices LLC, USA). [14] Untreated cells were taken as control with 100% viability and cells without addition of MTT were used as blank. Based on the results from above assay, cells were treated with test formulations petroleum latex of *Calotropis Gigantea* loaded hyalurosomes (CGHGF) and CG-Carbopol gel at 1 to 50µM concentration range. After 24h exposure of cells to different formulations determined the effect of formulations on cell viability.

Melanin Content Assay

B16F10 cells were seeded in two 12 well plates at a density of 1×10^5 cells/well and incubated for 24 hrs. For evaluating the effect of first treatment (cells were treated once), the media was replaced by test formulations equivalent to 5 µM concentration of each drug followed by UV exposure for groups. After 48 hrs. of exposure to formulations, cells were processed for further steps. For another 12-well plate, second treatment was also given (cells were treated twice) and similar procedure was followed as per first treatment. Media from the first treatment was replaced by fresh media containing formulations at 48 hrs. (followed by UV exposure for PSR groups) and incubated for additional 48 hrs. After 48 hrs. exposure (for first treatment) and 96 h exposure (for second treatment), cells were washed twice with PBS and lysed by incubation in lysis buffer (PBS with 1% Triton X-100) at 4 °C for 20 min. [15]

The lysates from both the plates were centrifuged separately at 14000 rpm for 15 min to collect the pellet. Supernatants were processed for tyrosinase activity determination whereas the cell pellet is dissolved using 1N sodium hydroxide (NaOH) containing 10% DMSO for 1h at 80°C to solubilize the melanin. The protein estimation was performed using Bradford's assay and bovine serum albumin was taken as a standard. Aliquots containing same amount of protein were taken and volume was made up to 100µL with NaOH solution. Absorbance was measured for samples at 366 nm and melanin content was calculated from a standard curve using synthetic melanin.

Tyrosinase Activity Assay

Cell supernatant obtained from the above procedure was analysed using Bradford's assay for its protein content. Samples containing same amount of protein were taken, volume was made up to 100µL with lysis buffer and then 0.1% L-DOPA solution prepared in PBS (100 µL) was added to it. The plate was incubated at 37°C for 1h and the dopachrome was monitored by measuring the absorbance at 475 nm. [16]

Cell Free Tyrosinase Assay

A cell-free assay system was used to test direct effects of CGHGF on tyrosinase activity. About 130 µl of sample dilutions prepared from test formulations at 1 to 50 µM concentration range were mixed with 20 µl of mushroom tyrosinase (1000 units) and 100 µl of L-DOPA solution (2 mg/ml). The assay mixtures were incubated at 37 °C for 20 min and absorbance of dopachrome was measured at 475 nm in a microplate reader. The mushroom tyrosinase activity was calculated and compared with control.[17]

Fontana-Masson Silver Staining

Cells were seeded in a 12 well plate at a density of 5×10^4 cells/well in the respective media and incubated for 24 h. Then the media was replaced by CGHFG formulations (5 µM equivalent drug concentration), followed by UV exposure and incubated for 24 h. Later, the cells were washed and fixed in 4% v/v formalin followed by staining with 2.5% w/v ammonical silver nitrate (temperature maintained at 56°C). The plate was incubated for 1hrs. and then washed with distilled water. Then 5% w/v sodium thiosulfate solution was added, incubated for 5 min and washed with distilled water. Later, the cells were stained with nuclear faster red solution for 5 min and washed again. Finally, after dehydration with ethanol the wells were washed with xylene twice. Then the plate was observed under phase contrast microscope for visualization of melanin pigment in wells**.** [18]

RESULTS AND DISCUSSION

FTIR analysis

The functionality of the Latex compounds was characterized by FTIR spectroscopy (Figure 1). The FTIR spectrum of pure Latex (A), Petroleum ether portion of latex (B), and methanol portion of latex (C) was determined. A broad peak at 3253 cm^{-1} was noted, this is response to the OH stretching frequencies of uscharin, calotropin, lubeol, and b-amyrin. High

intense broad peaks were appeared at 1628 cm^{-1} of di-ketonic bonds. The peaks at 1065 cm^{-1} corresponded to the C-N stretching bonds of aliphatic amines. The Petroleum ether soluble lax was identified to determine the characteristics of the compounds based on FTIR spectrum (Figure 1B) and compared with FTIR spectrum of pure latex (Figure 1A). The OH stretching frequency peak was not present, confirming that polar compounds were not present in the Petroleum ether portion. Other characteristic peaks indicated a COOH stretching vibration at 2975 cm^{-1} and bending vibration at 1320 cm^{-1}. Polar solvent soluble compounds showed a broad peak at 3375 cm^{-1} because of the OH group of the polar molecules (Figure 1C).

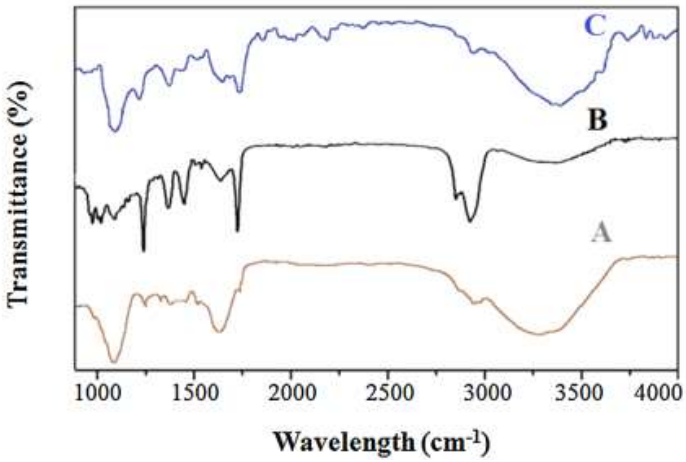

Figure 1: FTIR spectra of samples: (A) pure latex; (B) Petroleum ether latex; (C) methanol latex

Fluorescent studies

The Fluorescent studies of latex of *Calotropis Gigantea* by UV spectrophotometry stated that at visible-light the latex has shown the Yellowish white, at Short UV light (252 nm) Light yellow colour is shown and at Long UV light (366 nm) Alice blue colour is seen. The Petroleum ether latex at Visible light has shown the light cream, at Short UV light (252 nm) Yellowish Green colour is shown and at Long UV light (366 nm) cream colour is seen. The methanol latex at Visible light has shown the light yellow, at Short UV light (252 nm) Yellowish Green colour is shown and at Long UV light (366 nm) brown colour is seen.

The *Petroleum ether latex of Calotropis Gigantea* contain the lupeol, beta-amyrin (Water fraction of latex), alpha-calotropeol, and beta-calotropeol used as drug for the Topical Nano delivery of therapeutics and used as drug for treatment of vitiligo by topical nanogel delivery system.

Development of Hyalurosomes nanogel formulation

The rationale for selection of hyaluronan as a key ingredient in the hyalurosomes formulation was that it is a natural non-irritant, allows deep skin penetration, and is self-gelling. Moreover, because there is a direct relationship between the viscosity of hyaluronan and its concentration, increasing its concentration from 0.2% to 3% could successfully convert liquified-HS into more viscous hyalurosomes nanogel formulation. The optimized CGHGF formulation was utilized for the pharmacological evaluations.

PHARMACOLOGICAL EVALUATION

Evaluation studies on B16F10 cell line

The study constraints in developing animal model for vitiligo, *in vitro* assessment was performed to evaluate the efficacy of CGHGF. Spontaneous and induced animal models developed to evaluate new treatments of vitiligo have their distinct advantages and disadvantages. Induced models may not demonstrate initiating events of vitiligo while spontaneous models which develop in a more physiologic manner can be time-consuming and costly due to low incidence of disease. Hence, mouse melanoma cells (B16F10) which address the experimental questions of present study were chosen to evaluate CGHGF formulations.

Cell viability assay

This study was performed to decide on the concentration that exhibits maximum stimulatory effect on pigmentation parameters with minimal cytotoxic effect. Initially, this assay was done using Petroleum latex of *Calotropis Gigantea* as drug (0.25%w/w, 5 mg) to determine IC_{50}, the concentration which inhibits 50% cell viability and in-turn ascertain working concentration range. From this assay, CGHGF showed 68.71±2.76% cytotoxicity, Petroleum latex of *Calotropis Gigantea* solution showed 22.10±9.63% cytotoxicity and CG-Carbopol Gel showed 52.10±9.63% cytotoxicity at 50µM concentration (Figure 2A). Further, MTT assay was performed to evaluate the cytotoxic effect of different test formulations i.e., Latex solution, test formulations (CG-Carbopol Gel) and Petroleum latex of *Calotropis Gigantea* loaded-hyalurosomes (CGHGF) on B16F10 cells. All the formulations were tested at respective drug concentration ranging from 1 to 50 µM (this concentration range was selected based on the results of initial cell viability assay). Dose dependent increase in cytotoxicity was observed with all the formulations and the cytotoxicity observed with drug loaded

CGHGF was slightly higher compared to respective drug solution (Figure 2B). A concentration which shows therapeutic efficacy with minimal toxicity has to be selected for further studies.

Figure 2 (A): Cell Viability study of Petroleum latex of *Calotropis Gigantea,* CGHGF and CG-Carbopol gel
Figure 2(B): Effect of different nano-formulation on cell viability, B16F10 cells were exposed to different formulation i.e., Petroleum latex of *Calotropis Gigantea,* CGHGF and CG-Carbopol gel in concentration range 1-50 µM. All dare represent mean±SD (n=3).

The Latex solution has shown the CG-Carbopol gel showed 56.11±11.55% cytotoxicity at 50 µM concentration of Latex while showed CGHGF 82.95±11.61% cytotoxicity at 50 µM concentration of latex. Low drug concentrations i.e., 1 µM and 5 µM showed minimal cytotoxicity with all the formulations. Latex solution showed about 12.24±7.37% and CGHGF showed 32.5.10±0.46% cytotoxicity at 5 µM concentration. CG-Carbopol gel showed 22.6±1.56% cytotoxicity at 5 µM concentration (Figure 2B). As above 60% of cells are viable in all the formulations at 5 µM equivalent drug concentration, this was selected as working concentration for further studies. The *Petroleum latex of Calotropis Gigantea* showed dose dependent therapeutic effect by stimulation of melanogenesis and tyrosinase activity at a concentration ranging from 1 to 10 µM.

Melanin And Tyrosinase Activity Assay

Petroleum latex of *Calotropis Gigantea* may acts in vitiligo by stimulating melanisation, increased synthesis of tyrosinase via cAMP activity and by photo-polymerization of melanogenic precursors. In this study, the effect of Latex solution, test formulations (CG-Carbopol Gel) and Petroleum latex of *Calotropis Gigantea* loaded hyalurosomes (CGHGF) on melanin content and tyrosinase activity was determined. These parameters were evaluated in terms of first treatment (cells were treated once) and second treatment (cells were treated twice).
Melanin is a natural pigment that plays a vital role in skin pigmentation and destruction of melanocytes leads to depigmentation which is the pathological hallmark of vitiligo. Compared to control (85 relative melanin content), Petroleum latex of *Calotropis Gigantea* solution showed an increase in melanin content by 1.20 fold upon first treatment ($p<0.05$) and 1.20 fold upon second treatment ($p<0.05$). CGHGF is showed a 2.25 fold increase in melanin with first treatment ($p<0.0001$) and a 2.89 fold increase with second treatment ($p<0.0001$). This showed that effect of CGHGF showed enhanced stimulation of melanin levels compared to Petroleum latex of *Calotropis Gigantea*. CGHGF showed a significant difference in melanin proportion between first and second treatment levels ($p<0.05$) which indicates dose dependent elevation of melanin content.
For Petroleum latex of *Calotropis Gigantea* solution and CG-Carbopol Gel, significant increase in melanin content was observed in comparison with control. This confirmed that has either inhibitory or stimulatory effects on melanin content at 5 µM working concentration. For CG-Carbopol Gel, there was 2.05 fold increase in melanin content with first treatment ($p<0.01$) while 2.64 fold increase was observed with second treatment ($p<0.01$) (Figure 3A).

Figure 3(A): Effect of different Nano-formulation on relative melanin content in B16F10 cells.

Tyrosinase is considered as the key determinant of pigmentation and as rate limiting enzyme for melanin synthesis. CGHGF showed a significant increase in tyrosinase activity by 2.64 fold with first treatment ($p<0.001$) and 2.72 fold increase with second treatment ($p<0.0001$) compared to control while a very slight increase in tyrosinase activity was observed with Petroleum latex of *Calotropis Gigantea* solution. (Figure 3B) This ensured enhanced therapeutic effect of PUVA with CGHGF compared to Petroleum latex of *Calotropis Gigantea* solution.

Figure 3(B): Effect of different Nano-formulation on relative tyrosinase activity in B16F10 cells.

Figure 3(C): Cell free tyrosinase assay to determine the direct effect of different formulation on tyrosinase activity.

In a cell free system, tyrosinase assay was performed with mushroom tyrosinase and LDOPA to determine the direct effect of Petroleum latex of *Calotropis Gigantea* formulations on tyrosinase activity. No significant difference was observed in tyrosinase activity with any of the formulation groups compared to control (Figure 3C). This showed that Petroleum latex of *Calotropis Gigantea* did not have direct effects on tyrosinase activity. This is in support with a previous report which stated indirect effect of PSR on tyrosinase synthesis via cAMP that further increases tyrosinase enzymatic activity.

Fontana-Masson Silver Staining

This staining is performed to visualize melanin pigment in cells exposed to *Petroleum latex of Calotropis Gigantea* CGHGF formulations.

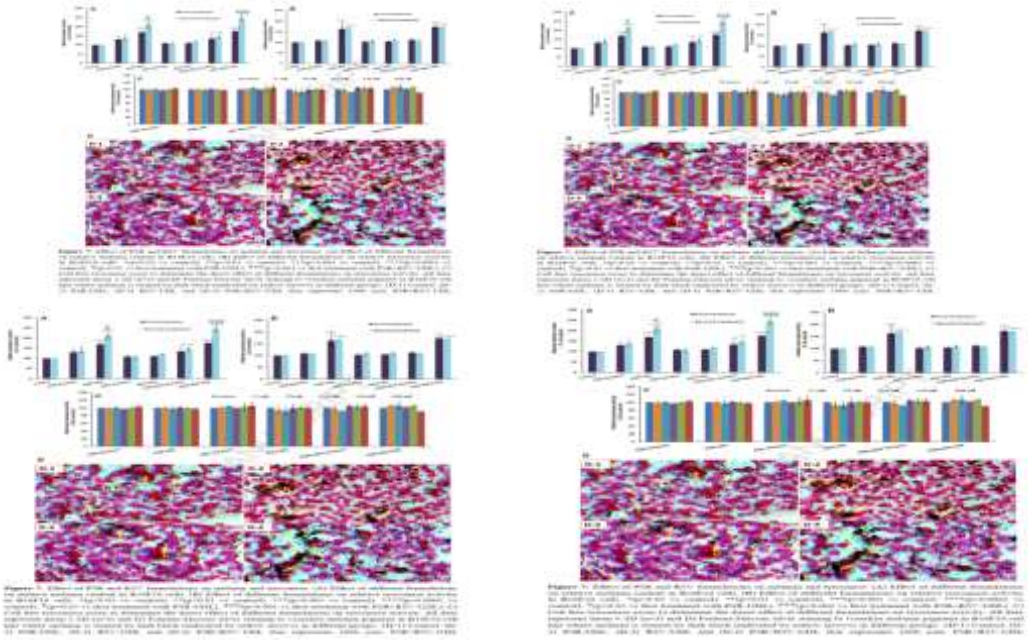

Figure 3D: Fontana-Masson silver staining to visualize melanin pigment in B16F10 cell line where melanin is stained by dark black (indicated by arrows). D-1= Control, D-2= Petroleum latex of *Calotropis Gigantea* solution, D-3= CG-Carbopol Gel and D-4= CGHGF.

It is a histochemical technique based on oxidation of melanin and reduction of silver resulting in a black stain that can be visualized under microscope. Compared to the control (Figure 3D-1), the amount of melanin granules was significantly increased and stained in Petroleum latex of *Calotropis Gigantea* solution (Figure 3D-2), CG-Carbopol Gel (Figure 3D-3) and CGHGF (Figure 3D-4) groups (indicated by yellow arrows in figure). The obtained results were in agreement with the results obtained with melanin content and tyrosinase assay.

CONCLUSION

The currently marketed treatments for vitiligo have shown limited effectiveness, which has created a substantial demand for novel vitiligo treatments. The topical *Petroleum latex of Calotropis Gigantea* loaded-hyalurosomes (CGHGF) investigated in this study showed outstanding properties for effective treatment of vitiligo through permitting its delivery into human skin with a high deposition level. In conclusion, *Petroleum latex of Calotropis Gigantea* loaded-hyalurosomes (CGHGF) hyalurosomes investigated in this study showed promising skin permeation and deposition properties that should be highly useful for clinical treatment of numerous skin-disorder**.**

Conflict of Interest: Nil.

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