



Received on 24 August, 2016; received in revised form, 15 October, 2016; accepted, 06 December, 2016; published 01 March, 2017

## DESIGN AND DEVELOPMENT OF EMULSION-BASED CLEAR COMPLEXION SKIN WHITENING CREAM USING *GLYCYRRHIZA GLABRA* (LICORICE) ROOT EXTRACT AND *VATERIA INDICA* (WHITE DAMMAR) BARK EXTRACT BY SKIN MELANIN INHIBITORY PATHWAY

N. Kirubakaran <sup>\*1</sup>, Thiagarajan <sup>2</sup>, Chandrika Mahendra <sup>1</sup>, Sandeep Varma <sup>2</sup>, Vijaya Kumar <sup>3</sup>, Virendra Tripathi <sup>1</sup> and Babu <sup>3</sup>

Department of Personal Care Formulations <sup>1</sup>, Department of Cell Biology <sup>2</sup>, Department of Phytochemistry <sup>3</sup>, The Himalaya Drug Company, Makali, Bangalore 562162, Karnataka, India.

### Keywords:

Emulsion, Skin lighting,  
Physical sunscreens, Skin whitening,  
Melanin, Rheology

### Correspondence to Author:

**N. Kirubakaran**

Research and Development, The  
Himalaya Drug Company, Makali-  
Bangalore, Karnataka, India.


**Email:** Kirubakaran\_14@yahoo.co.in

**ABSTRACT:** The objective of this study was to design and develop a skin lighting cream for improved skin whitening using herbal extracts from *Glycyrrhiza glabra* (Licorice) root and *Vateria indica* bark. The carrier cream base was developed by emulsion technique to deliver skin whitening property by holding the natural herbal actives such as *G. glabra* root extract and *V. Indica* bark extract as well as physical sun protecting agents such as titanium dioxide for the skin whitening properties. The emulsion cream was designed and optimized to deliver the skin lighting property by the combination of *G. glabra* root extract and *V. Indica* bark extract. This unique composition helps to provide the melanin inhibition effect through cellular melanin inhibition pathway. The optimized emulsion cream formula was evaluated as per ICH stability guidelines (real time, 30°C and 65% Relative Humidity (RH) and accelerated, 45°C and 75% RH) to ensure emulsion cream stability and shelf life of the product. The optimized and stabilized formula flow behaviours were determined by various rheological studies such as flow curve measurements (viscosity vs. shear rate), amplitude sweep measurement (linear visco elastic range), frequency sweep measurements (stability test), amplitude sweep measurements (spreadability test), and thixotropic test. The performance of the final product was measured by skin melanin inhibition property and the results observed indicated better skin lighting compared with control. The safety of the final product was measured by Netral Red Uptake NRU-assay using NIH3T3 cell lines and the CTC<sub>50</sub> value of the product was found to be >1000µg/ml at 24 h study period.

**INTRODUCTION:** Skin complexion is a characteristic that is affected by many factors. Various genes, nutrition, and environmental factors determine the colour of the skin. One of the most notable components of skin that contributes to complexion is a pigment known as melanin.

Melanin is a pigment that is produced by cells known as melanocytes in the skin of most animals including humans. This pigment comes in different shades, depending on the genetic makeup of the individual. Melanin comes in two basic forms and can range from yellowish-red to dark brown. Eumelanin is the most common form of melanin and is brownish.

The other basic form is called pheomelanin, which produces reddish-brown colour that is often associated with freckles and red hair. The production of melanin in an individual is determined by several factors.

<b>QUICK RESPONSE CODE</b> 	<b>DOI:</b> 10.13040/IJPSR.0975-8232.8(3).1210-19
	Article can be accessed online on: <a href="http://www.ijpsr.com">www.ijpsr.com</a>
<b>DOI link:</b> <a href="http://dx.doi.org/10.13040/IJPSR.0975-8232.8(3).1210-19">http://dx.doi.org/10.13040/IJPSR.0975-8232.8(3).1210-19</a>	

- **Exposure to UV radiation:** Melanin is produced as a response to UV radiation to prevent damage to the DNA in the integument. Individuals, who are exposed to UV light, such as that from the sun, will produce more melanin for protection.
- **Genetic makeup:** Different ethnicities and cultures are genetically predisposed to produce particular shades and amounts of melanin. This is essentially one of the primary indicators used in determining race in the human population. It is important to note that this is, and has historically been, a controversial form of human identification.
- **Size of melanocytes:** Melanocyte size varies in different individuals and may lead to a difference in the amount of melanin produced per cell.
- **Disease conditions:** Several diseases may affect melanin production, including albinism, a genetic inability to produce melanin, and vitiligo, a progressive loss of melanocytes.

All these factors play a role in the production and expression of melanin in the skin.

Here the objective is to design and develop a skin lighting cream for improvement of skin whitening using herbal extracts from *Glycyrrhiza glabra* (Licorice) root and *Vateria indica* bark. The carrier cream base to be designed by emulsion technique to deliver skin whitening property by holding the natural herbal actives such as *G. Glabra* root extract and *V. indica* bark extract as well as physical sun protecting agents such as titanium dioxide for the skin whitening properties. The performance of the final product to be measured by skin melanin inhibition assay and the safety of the final product to be measured by Netral Red Uptake NRU-assay using NIH3T3 cell lines to find out the safety aspects.

## MATERIALS AND METHODS:

### Materials:

**Gelling and active materials:** Polyacrylic/ copolymer and acrylates C<sub>10-30</sub> alkyl acrylates cross-polymer and polysaccharide gumming agent

were purchased from Arihant Innochem Pvt Ltd (Mumbai, Maharashtra, India). *Glycyrrhiza glabra* root extract and *Vateria indica* bark extract were prepared in-house (The Himalaya Drug Company, Bangalore, Karnataka, India).

**In vitro cell culture study materials:** The cell culture experiments were performed using mouse melanoma cells (B16F10) and mouse embryo fibroblasts (NIH3T3), which were obtained from the National Centre for Cell Science (NCCS; Pune, Maharashtra, India). They were maintained in DMEM-high glucose (Sigma, Bangalore, Karnataka) with 10% fetal bovine serum, 1% antibiotic solution-penicillin (100 U/ml) and streptomycin(100 µg/ml) (Hi-Media), and 2.0 and 2.5g/L sodium bicarbonate (Sigma) using 25cm<sup>2</sup> polystyrene flask (Tarsons, Kolkata, West Bengal, India) under the atmosphere of 5% CO<sub>2</sub> at 37°C with 95% humidity.

**Polyacrylic/copolymer:** Acrylates copolymer is a copolymer of two or more monomers consisting of acrylic acid, methacrylic acid, or one of their simple esters (Wenninger *et al.*, 2000). The smallest or primary units of acrylates copolymer are individual particles < 1 µ in diameter, which partially fuse to form agglomerates ranging in size from approximately 20 to 80µ; agglomerates are held together by electrostatic forces and mechanical entanglement to form larger aggregates of 200 to 1200µ (Klein and Disapio, 1989).

**Polysaccharide gumming agent:** Microbial polysaccharide gums may be ionic or non ionic and are primarily linear polysaccharides to which side chains of varying length and complexity are attached at regular intervals (Ma *et al.*, 2002). Most microbial polysaccharide gums are linear hetero polysaccharides consisting of three to seven different monosaccharides arranged in groups of 10 or less to form repeating units. The monosaccharides may be pentoses, hexoses, amino sugars, or uronic acids. For example, xanthan gum is a polysaccharide produced by a pure culture fermentation of a carbohydrate with *Xanthomonas campestris* and is composed of glucose, glucuronic acid, 6-acetylmannose and 4, 6-pyruvylated mannose residues as seen in **Fig. 1**



MTT (5 mg/ml) was added and the cells were incubated for 4 h at 37°C. The supernatant was removed and 100 µl of dimethyl sulfoxide (Merck, Mumbai, Maharashtra, India) was added to each well to dissolve the formazan product. Absorbance was determined at 540 nm spectrophotometrically using an ELISA reader (Synergy HT multi-detection micro plate reader). The CTC<sub>50</sub> values of CCW-F2 were calculated and the nontoxic concentrations were taken for further experiment.

**Melanin inhibition assay:** The mouse melanoma (B16F10) cells were seeded in 40 mm petriplates (1×10<sup>5</sup> cells/well) using the DMEM medium supplemented with 10% FBS for 24 h. The cells were treated with various concentrations of CCW-F2 and 100 µM concentration of forskolin for the stimulation of melanin for 72 h and detached by 0.05% trypsin-EDTA. The cell pellets were mixed in 120 µl of 1N NaOH for 1 h at 65°C and melanin concentration was determined spectrophotometrically 405 nm using an ELISA reader<sup>13, 14</sup>. For melanin content analysis, kojic acid was used as a reference standard.

**Preparation of root extract of *Glycyrrhiza glabra* and bark extract of *Vateria indica*:** The roots of *Glycyrrhiza glabra* and bark extract of *Vateria indica* were purchased from a local vendor in Bangalore, India, in the month of February 2012, and voucher specimen was preserved by

Pharmacognosy Department, R&D Center, The Himalaya Drug Company. The sample was washed with fresh water, shade dried, and powdered in a pulverizer. The dried and powdered material (each 100 g) was extracted separately by stirring with 100mL of hydro alcohol under hot extraction in 100 ml for 6 h in a soxhlet, and concentration was done under vacuum.

## RESULTS AND DISCUSSION:

**Stability study:** Stability studies were conducted as per the ICH guidelines to ensure the final optimized product stability with various conditions such as real-time (30°C and 65% RH) and accelerated conditions (45°C and 75% RH) for 6 months using Thermo lab stability chambers (Thermo lab, Thane, Maharashtra, India) Lab scale validation was performed in 3 batches namely CCW-PF/18, CCW-PF-19, CCW-PF-20 to study the stability of the final optimized formula.

**Viscosity study:** Viscosity is an important parameter to predict the consistency of the product. The viscosity was estimated using the viscometer (T-96 spindle at 1.00 rpm). The product stability was assessed initially and at first, second, third and sixth months under accelerated stability (45°C and 75% RH) and real-time stability conditions (30°C and 65% RH). The results are shown in Fig. 2.

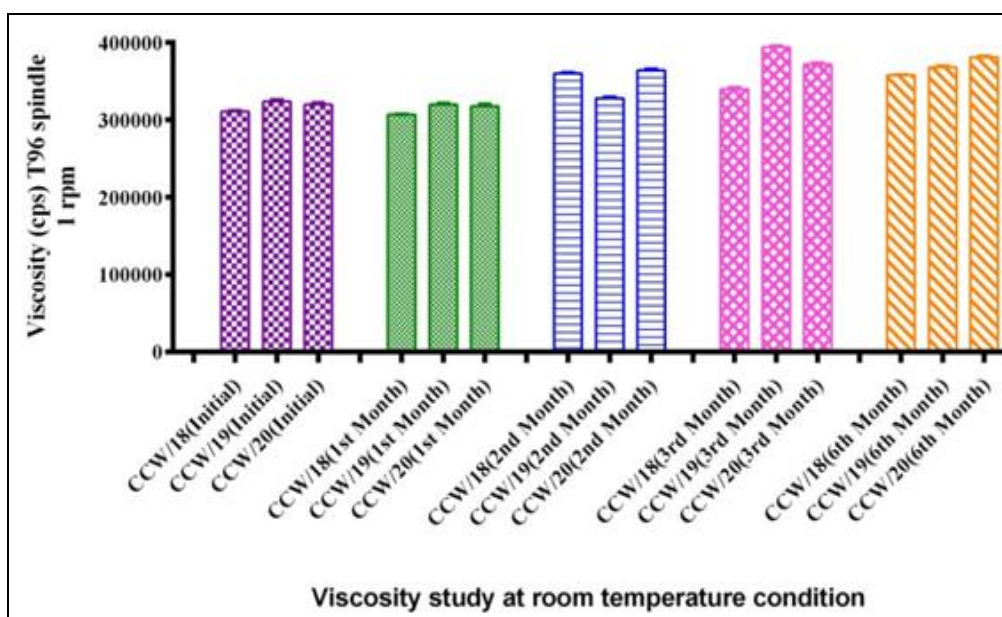


FIG. 2: VISCOSITY DETERMINATION UNDER REAL-TIME CONDITIONS (30°C AND 65% RH)

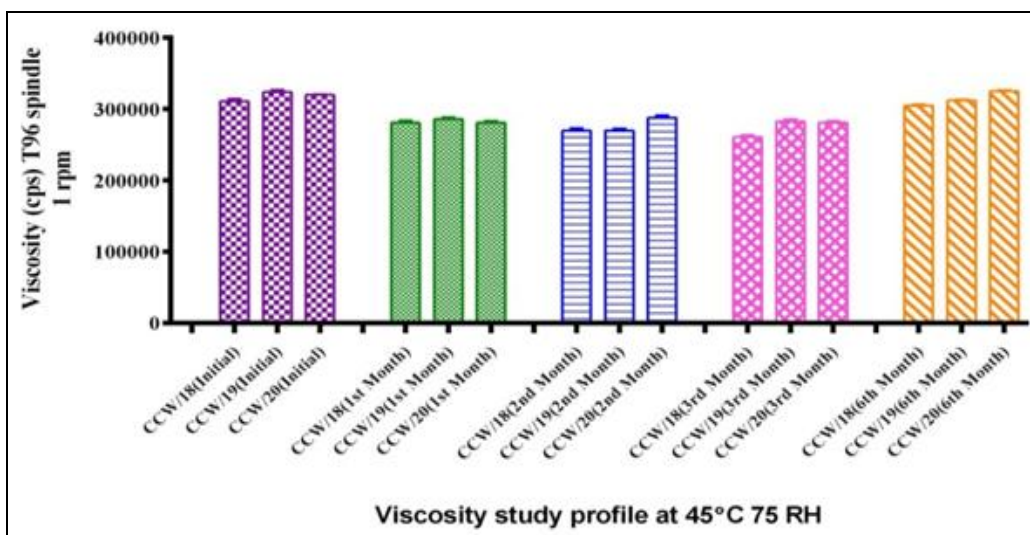


FIG. 3: VISCOSITY DETERMINATION UNDER ACCELERATED CONDITIONS (45°C AND 75% RH)

**pH determination:** pH was determined using the pH meter under real-time (30°C and 65 % RH) and accelerated conditions (45°C and 75% RH) at first,

second, third and sixth months. The results are shown in **Fig. 4** and **5**.

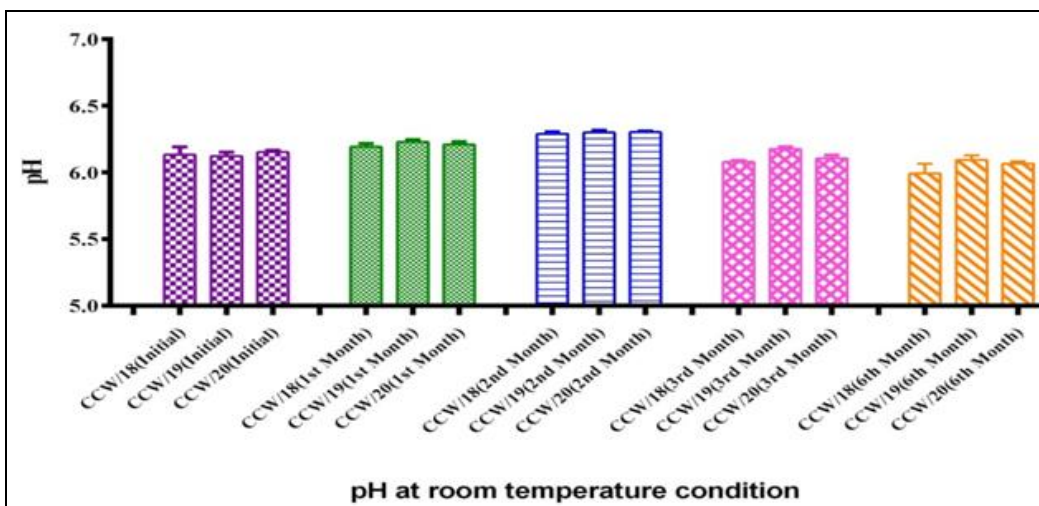


FIG. 4: pH DETERMINATION UNDER REAL-TIME CONDITIONS (30°C AND 65% RH)

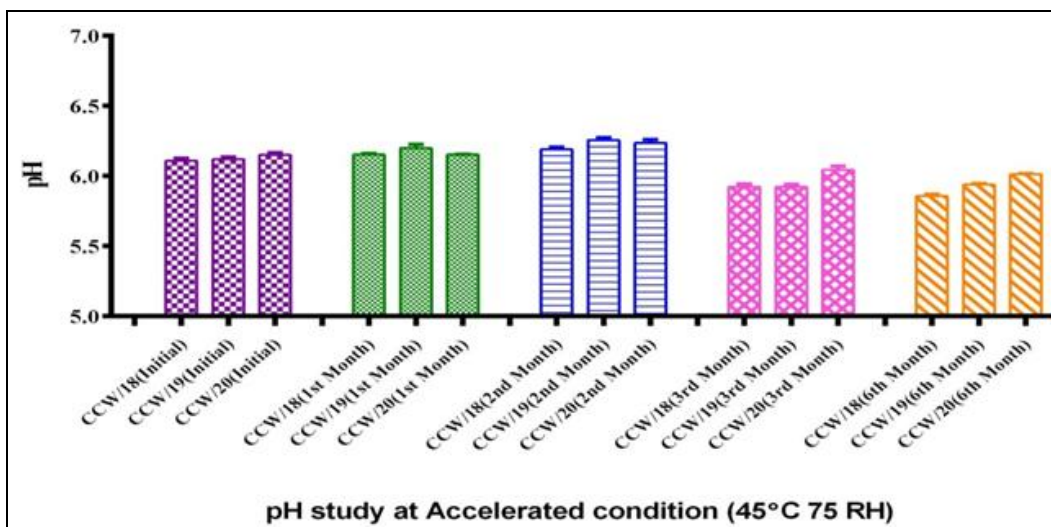
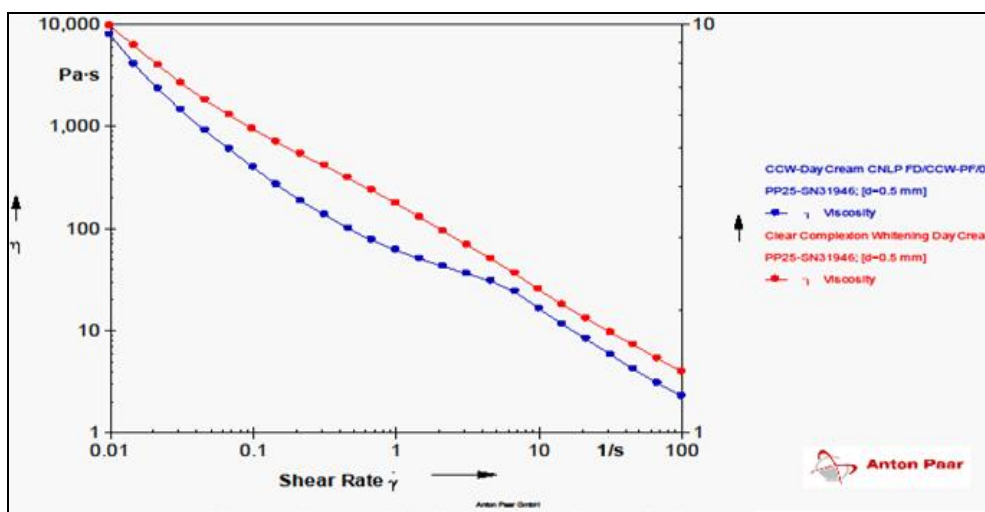


FIG. 5: pH DETERMINATION AT ACCELERATED CONDITIONS (45°C AND 75% RH)

**Rheological behaviour:** Rheology is the flow behaviour of the product. The flow curve, amplitude sweep, frequency sweep, and thixotropic properties were studied to understand the product behaviour with respect to flow properties, regaining properties, strain rate etc. The comparative rheological studies were performed with F1 (clear complexion whitening day cream1) and F2 (CCW-DAY CREAM FD/CCW-PF/09) formula.

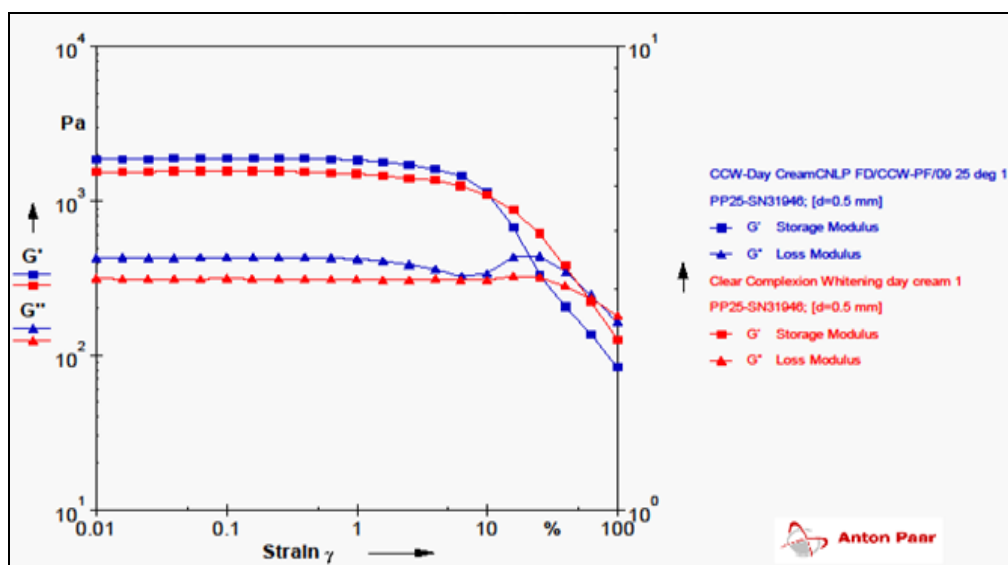
**Flow curve measurements:** The final product sample was analyzed for flow behaviour in the shear rate range of 0.01 to 100 1/s, which helped observe the changes in viscosity of the samples as a function of shear at 25°C (rotational mode). Shear thinning behaviour was observed and the results are shown in **Fig. 6**.



**FIG. 6: FLOW CURVE MEASUREMENTS (SHEAR RATE VERSUS VISCOSITY)**

**Amplitude sweep measurement (linear visco-elastic range):** Amplitude sweep from 0.01 to 100% strain rate was performed at constant angular frequency of 10 rad/s to determine the limit of linear visco-elastic (LVE) range. The results indicated that below this limiting value,  $G'$  (elastic modulus) and  $G''$  (loss modulus) curves remained at a constant plateau; the structure of the sample

showed stable curve under these conditions. In oscillatory mode the amplitude sweep showed that the limit of the LVE range for clear complexion whitening day creams (F1 & F2) were around 1.5%. The amplitude sweep curve showed that  $G' > G''$ , which implied that all the above samples (F1 & F2) have good emulsion strength. The results are shown in **Fig. 7**.

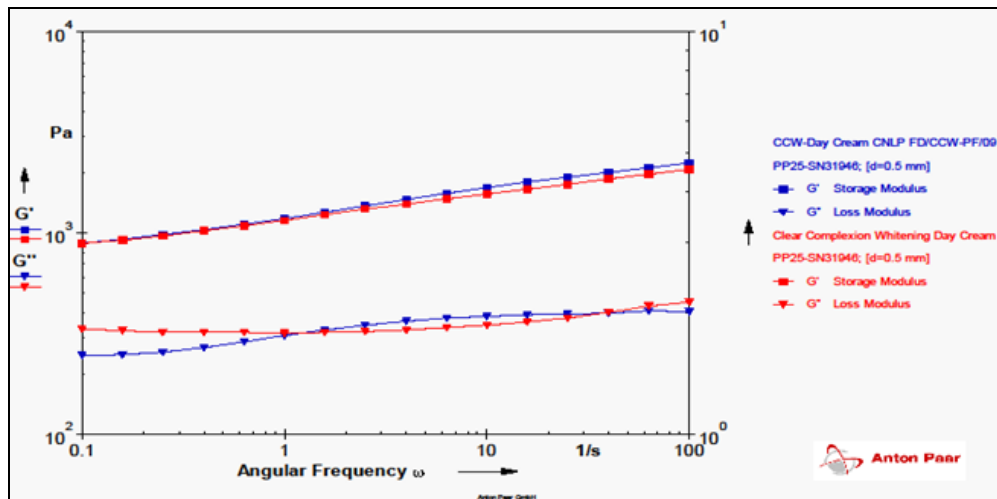


**FIG. 7: AMPLITUDE SWEEP MEASUREMENT (LINEAR VISCOELASTIC CURVES)**

**Frequency sweep measurements (stability test):**

After determining the LVE range, frequency sweep was carried out for each sample in the range of 0.1 to 100 rad/s by keeping strain rate (according to LVE range) constant to find out the visco-elastic behaviour and long-term storage stability of the cream samples with time (oscillatory mode). The samples are generally stored at 25°C to simulate the practical condition and frequency sweep experiments were performed at this temperature. As creams are semi-solid emulsions (dispersions),

their long-term stability at rest can be analyzed using  $G'$  values at low frequencies. The  $G'$  and  $G''$  values are compared to characterize the structure of the final product. The frequency sweep curves clearly showed that all the samples have  $G' > G''$  in whole frequency range, which indicates that all the samples have good structural strength. In the low-frequency region, the decrease in  $G'$  slope was less, which indicates good stability and shelf life of both the samples. The results are shown in **Fig. 8**.

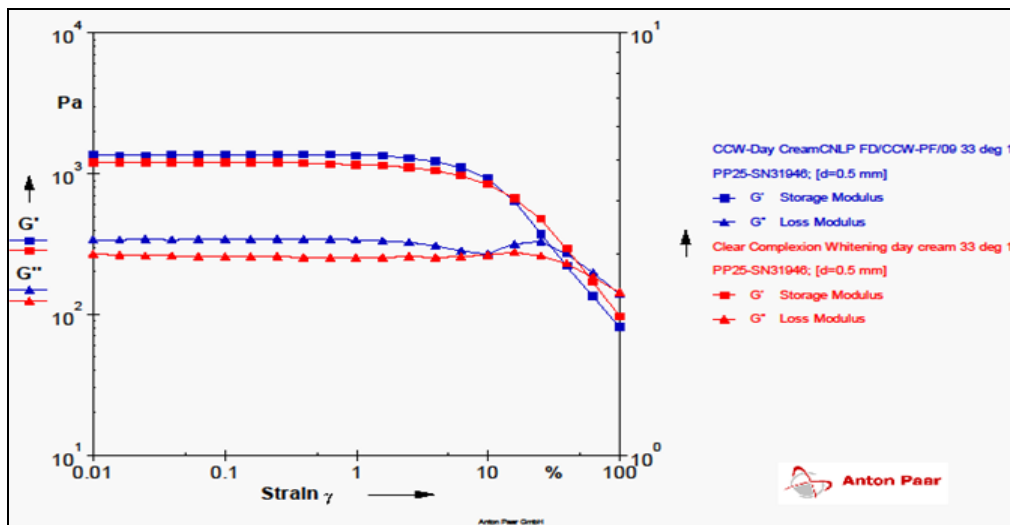


**FIG. 8: FREQUENCY SWEEP (STABILITY TEST) MEASUREMENTS**

**Amplitude sweep measurements (spreadability test):**

The temperature-dependent structural strength and spread ability test were carried out for amplitude sweep in strain range of 0.01 to 100% with a constant angular frequency of 10 rad/s at constant temperature of 33°C (to simulate the practical condition of the cream when it is applied to outer body parts). In the LVE range (ie, at very

low deformations), all the samples were observed to show  $G' > G''$ , which indicates that the elastic modulus is higher than viscous modulus (i.e., good emulsion strength of the samples). The  $G'$  curves show that both the samples spread smoothly and consistently with a similar curve pattern, which approves its spread ability. The results are shown in **Fig. 9**.

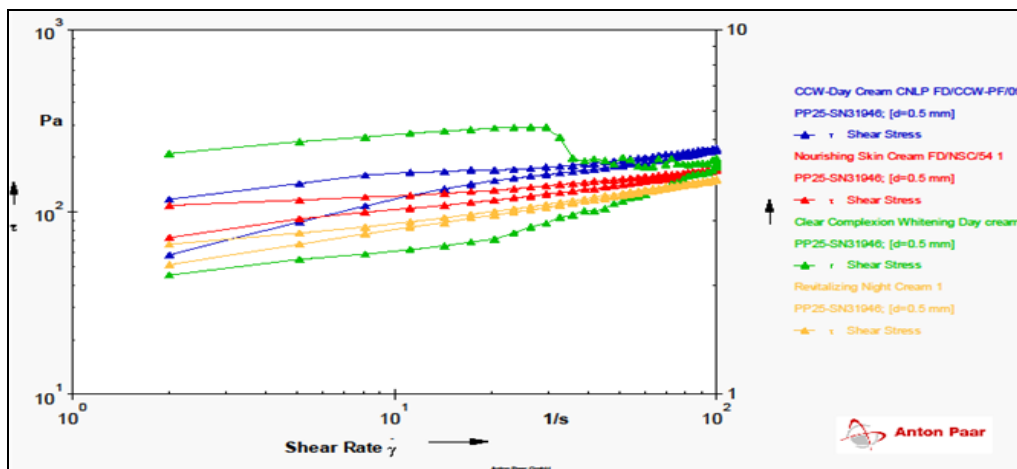


**FIG. 9: AMPLITUDE SWEEP (SPREADABILITY TEST) MEASUREMENTS**

**Thixotropic test:** The thixotropic test was done in 3 intervals.

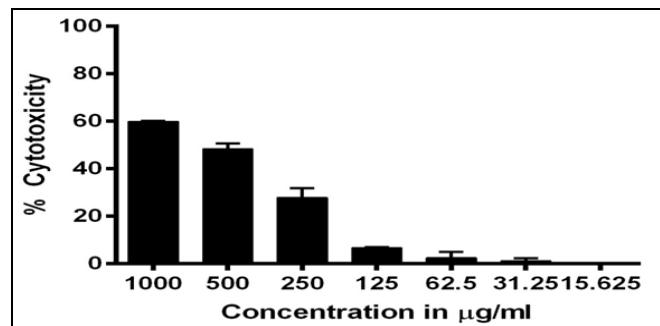
- In the first interval, the samples F1 (clear complexion whitening day cream 1) and F2 (CCW-DAY CREAM FD/CCW-PF/09) were analyzed with some market samples such as nourishing skin cream (FD/NSC/54) and revitalizing night cream at shear rate ramp of 2 to 100 1/s for 165 s.
- In the second interval, the samples were analyzed at constant shear of 100 1/s for 50 s.

- In the third interval, the samples were sheared down from 100 to 2 1/s shear rate. The cream samples were analyzed for structure regeneration (area under the curve), which yielded thixotropic indexes [Pa/(s.mL)] of 3451.89 and 19882 for the samples F2 (CCW-DAY CREAM (FD/CCW-PF/09) and F1 (clear complexion whitening day cream1), respectively. The thixotropic test results are shown in **Fig. 10**.

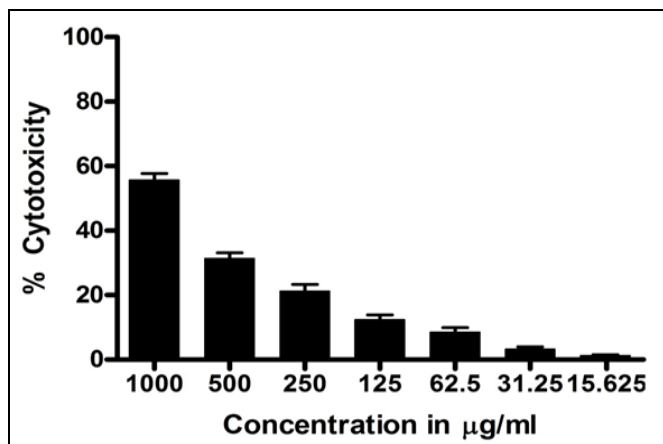


**FIG. 10: THIXOTROPIC TEST RESULTS**

**Cytotoxicity studies (MTT assay):** The cytotoxic activity of F2 (CCW-DAY CREAM) was analyzed by MTT assay. The results showed that increase in CCW concentration up to 1000 µg/ml did not reduce the cell viabilities in all tested concentrations in both B16F10 and NIH3T3 cells. The CTC<sub>50</sub> value of CCW-F2 was above 1000 µg/ml at 24 h, and the nontoxic concentration was taken for further study. The results are shown in **Fig. 11** and **12**.

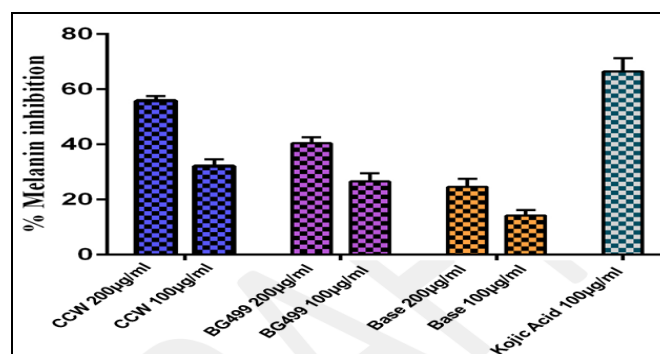


**FIG. 12: ASSESSMENT OF CYTOTOXIC ACTIVITY OF CCW-F2 USING NIH3T3 CELL LINES**



**FIG. 11: ASSESSMENT OF CYTOTOXIC ACTIVITY OF CCW-F2 USING B16F10 CELL LINES**

**Melanin inhibition study:**



**FIG. 13: MELANIN INHIBITORY PROPERTY OF CCW-F2 ON B16 F10 CELLS**



**Emulsion particle size distribution study:** The final optimized emulsion derived formula was subjected to particle size distribution study. The particle size distribution study was performed in

Malvern-Mastersizer-2000, United Kingdom. The results were observed at d(0.1) as 1.093µm, d(0.5) as 3.717µm and d(0.9) as 13.920 µm to ensure the particle size distribution and emulsion stability.

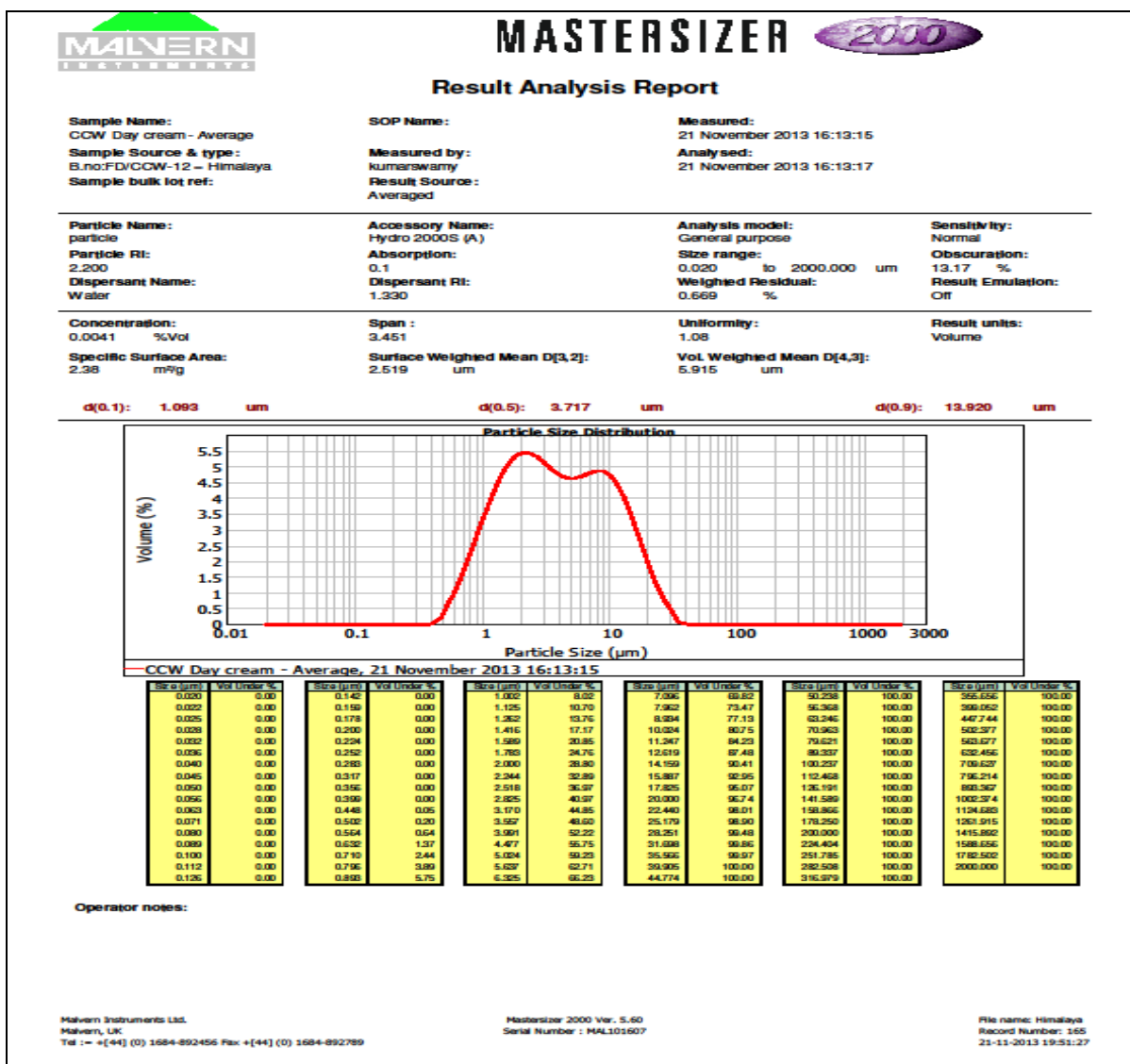


FIG. 14: EMULSION PARTICLE SIZE DISTRIBUTION

**CONCLUSIONS:** The emulsion based cream was designed and optimized using suitable polymers, stabilizing and emulsifying agents. The actives from *G. Glabra* root extract and *V. indica* bark extract in the cream inhibit melanin pigment and thus, help in skin lighting. The final optimized product (F2) was subjected to stability studies as per the ICH guidelines under both real-time (30°C and 75% RH) and accelerated conditions (45°C and 75% RH). The results showed better emulsion stability under both the conditions. pH study was performed to ensure the pH range and product stability. At all temperature conditions, the pH

range was observed to be between 5.86 (minimum) and 6.30 (maximum). The viscosity study revealed the range to be between 2,70,000 cps (minimum) and 3,94,000 cps (maximum). The CTC<sub>50</sub> value of CCW day cream (F2) was above 1000 µg/ml at 24 h. The melanin inhibitory study results highlighted the better skin melanin inhibition in melanocytes at the tested concentrations. Therefore, CCW day cream (F2) brings about melanin inhibition in skin cells and plays a vital role in improving complexion and whitening for better skin aesthetics.

**DECLARATION OF INTEREST:** The authors report no declarations of interest.

**ACKNOWLEDGMENTS:** This research was performed at The Himalaya Drug Company (Bangalore, Karnataka, India). We express our sincere gratitude to Dr Rangesh, Chief Scientific Officer, R & D Center and the Himalaya Drug Company, for his extensive support. We thank the Department of Phytochemistry (Mr Nishant), Skin care formulations (Krishna Bhama) and Analytical Department (Mr Sukumaran and Ms Akshatha) and Scientific Publications Department (Dr. Jayashree B. Keshav and Ms. Shruthi V. Kumar) of the Himalaya Drug Company for their support.

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### How to cite this article:

Kirubakaran, Thiyagarajan, Mahendra C, Varma S, Kumar V, Tripathi V and Babu: Design and development of emulsion-based clear complexion skin whitening cream using *Glycyrrhiza glabra* (licorice) root extract and *Vateria indica* (white dammar) bark extract by skin melanin inhibitory pathway. *Int J Pharm Sci Res* 2017; 8(3): 1210-19. doi: 10.13040/IJPSR.0975-8232.8(3).1210-19.

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