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Development of an Anti-Aging Poly Herbal Cream

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Abstract - The purpose of this research was to create a cream that has anti-aging properties by combining aqueous poly herbal extracts of four different plants: Rosa damascena flowers (RDW), Clerodendrum paniculatum leaves (CPW), Murraya koengii leaves (MKW), and Annona squamosa leaves (ASW). Using an enzyme-linked immunosorbent assay (ELISA), researchers were able to verify that human dermal fibroblast (HDF) cells do contain collagen-I. CB 01, CB 02, and CB 03 are the three custom blends of plant extracts that were created. Collagen-I levels were significantly higher in the extract mix (CB03) at 18.65 0.061ng/ml (p 0.05) than they were at 16.53 0.064ng/ml for 100g/ml of normal ascorbic acid. Following the development of five distinct carrier cream bases (F-1, F-2, F-3, F-4, and F-5), the optimal formulation was found to be F2 (PHY/AAC/01) with (CB03) active blend (PHY/AAC/02), which demonstrated a collagen-I content of 17.53 0.011ng/ml in HDF cells. Using a neutral red uptake assay using NIH3T3 cell lines, the in vitro cytotoxicity of compounds (CB 03) and (PHY/AAC/02) was determined to be above 1000 g/mL for a 24-hour study period. Physical and chemical parameters, such as pH and viscosity, were used to verify the final compositions' heat stability. Collagen-I gene expression was observed to be upregulated in the formulation (PHY/AAC/02) containing (CB 03) compared to the control and standard (market sample) formulations, suggesting that this ingredient may be useful for combating the ageing process and fostering the formation of a collagen matrix.

Keywords - Human Dermal Fibroblast (HDF) , Vitro Cytotoxicity ,Enzyme-linked Immunosorbent Assay (ELISA), Annona squamosa leaves (ASW) , Clerodendrum paniculatum leaves (CPW)

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INTRODUCTION

The skin plays a crucial role in protecting the body's interior organs from harmful environmental factors. The skin has three primary layers: the epidermis, the dermis, and the subcutaneous tissue. Keratinocytes are the major component of the epidermis, which serves to defend the skin from the environment. Humans are united in their pursuit of youthful, radiant skin, and they are willing to shell out serious cash to achieve this goal [1]. The skin's radiance and glow highlight crucial indicators of health and happiness.[2] Ageing causes a breakdown in the skin's barrier function, which lowers sebum production, skin pH, stratum corneum moisture, and prevents trans-epidermal water loss [3]. Skin ageing can be broken down into two categories: extrinsic and intrinsic. Sunlight, air and water pollution, tobacco use, and bad eating habits are all examples of extrinsic causes of ageing. Genetic causes and passing time are responsible for intrinsic ageing. Significant cause for alarm, extrinsic ageing is most noticeable on skin with visible signs of ageing such as wrinkles, laxity, brown patches, and uneven pigmentation [4].

Collagen is the most important of several types of fibrous proteins found in skin. Collagen, a protein found naturally in our connective tissue, is crucial to the health and pliability of our skin. Fibroblasts, granulation tissue, and epithelium are all replaced, and the process creates a cross-linking network at the site of injury. There are 16 main forms of collagens, with Collagen I, II, and III accounting for 80 percent of the body's total collagen content. Tendons, cartilage, and reticular fibres of the epidermis contain the anti-aging proteins collagen I and collagen III, respectively.

Plant extracts high in biomarkers including flavonoids, triterpenes, alkaloids, tannins, and other bio-molecules have been shown to trigger the process of skin rejuvenation. [5]. An in vitro study measuring collagen synthesis activity for Veronica officinalis extract was carried out to evaluate the efficacy of the specially prepared cream against wrinkles [6]. Anti-wrinkle and skin-healthy ingredients found in aloe vera, grapes, wheat, nettles, and camellia sinensis [7]. There were four plants, Annona squamosa, Clerodendrum paniculatum, Murraya koenigii, and Rosa damascena, chosen for their possible anti-aging properties, and those properties were the subject of the current study.

In various cultures, the leaves of the plant Annona squamosa (Annonaceae) are applied to wounds as a form of traditional medicine. Several in vitro studies, including the DPPH, nitric oxide, superoxide, and lipid peroxidase tests, have revealed antioxidant activity in Annona extracts from various species [8]. Extract of A. squamosa stimulates cell growth and collagen production in HD cultures.[9] For skin problems, you can use an extract of the leaves from the Clerodendrum paniculatum plant (Lamiaceae). Phenolics, steroids, flavonoids, terpenes, and volatile oils are some of C. Paniculatum's most prominently listed compounds [10].

In India, a common aromatic spice is the leaves of the plant Murraya koenigii (Rutaceae). Antioxidants and proteosome activity in M. koenigii have been reported. [11]. An alkaloid found in the leaves has been credited with anti-aging and anti-cholinesterase properties [12]. It has been observed that Rosa damascena (Family: Rosaceae) flower extracts, both fresh and dried, have antioxidant activity. Biomarkers for antioxidant activity have been found in R. damascena flowers, petals, and leaves. [13]. The blooms of the R. damascena plant include phenolic chemicals such as Gallic acid [14]. The purpose of this research was to assess the efficacy of a novel poly-herbal compound in reviving collagen and firmness in the skin. In vitro test of collagen content on human dermal fibroblast cells and confirmation by Collagen-I gene expression studies helped to justify the use of this poly-herb extract blend recipe as a new, natural solution for skin vitality.

MATERIALS AND METHODS

Materials

Plant materials: The C. Paniculatum (NPD/137/2014) foliage, the R. Damascena (NPD/686/2012) flowers, and the M. leaves of the koengii tree (NPD/86/2014) and A. squamosa leaves (Batch no. NPD/85/2014) were acquired from a regional market source and identified and authenticated by a professional botanist from The Himalaya Drug Company's Department of Pharmacognosy (Bangalore, India). The Himalaya Drug Company's Phytochemistry R&D Centre isolated the active ingredients from the various plants used (Bangalore, India).

Information for Researching Cells on a Petri Dish in the Lab: Human dermal fibroblasts (HDFs) and NIH 3T3 mouse embryo fibroblasts were used in the cell culture experiments. Both were purchased from the National Centre for Cell Science (NCCS; Pune, India). They were kept in a 5% CO_2 environment at 37 °C with 95% humidity in a 25cm2 polystyrene flask (Tarsons, Kolkata, India) with 10% foetal bovine serum, 1% antibiotic solution (penicillin (100 U/mL) and streptomycin (100 g/mL)) (Hi-Media), and 2.0 and 2.5 g/L sodium bicarbonate (Sigma).

Ingredients Used in the Cream's Formulation: For the cream's preparation, the following excipients were used: carbopol 980 (Lubrizol, USA); cetyl alcohol (Croda, Mumbai, India); cetostearyl alcohol (Croda, Mumbai, India); capryliccapric triglyceride (Subhash chemicals, Mumbai, India); glycerine (Godrej Industries, Mumbai, India); isopropyl myristate (Croda, Mumbai, India); (Zungbungler, USA). To make plant extracts and poly-herbal blends, the various plant components were rinsed with clean water, air-dried in the shade, and then ground to a powder in a pulverizer. Under hot extraction conditions, 600 mL of water was stirred with 100 g of dried and powdered plant material (each) for 6 hours. The total weight of the herb material used was 100 g. Extractions were concentrated using a Rota vapour (Make: Buchi; Model: R 215V) centrifuge operating at low pressure and high vacuum. After isolating the active components of the water extracts, three blends were created using varying permutations of the extracts; these were then given the designations (CB01), (CB02), and (CB03) based on their cyto-toxicity and collagen content, respectively. Table 1 details the extract yield.The C. Paniculatum (NPD/137/2014) foliage, the R. Damascena (NPD/686/2012) flowers, and the M. leaves of the koengii tree (NPD/86/2014) and A. squamosa leaves (Batch no. NPD/85/2014) were acquired from a regional market source and identified and authenticated by a professional botanist from The Himalaya Drug Company's Department of Pharmacognosy (Bangalore, India). The Himalaya Drug Company's Phytochemistry R&D Centre isolated the active ingredients from the various plants used (Bangalore, India).

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Table 1: Water Extractive Yields of selected plants

S.No.	Name of the plants	Part used	Extractive yield (%w/w)
1	*Rosa damascena*	Flowers	2.76
2	*Clerodendrum paniculatum*	Leaves	24.24
3	*Murraya koengii*	Leaves	7.58
4	*Annona squamosa*	Leaves	18.37

(CB01) was created by combining water extracts of R. damascena, C. paniculatum, M. koengii, and A. squamosa in a ratio of 1:2:2:1. This mixture of herbs was used to create (CB02) at a ratio of 1:1:2:2, and (CB03) at a ratio of 2:1:1:1. Collagen and cytotoxicity assays were performed on samples of (CB01), (CB02), and (CB03). There were five distinct cream bases produced (F-1, F-2, F-3, F-4, and F-5), with F-2 being the one that was finalised for future tests after a seven-day stress test. All following tests made reference to and recorded F-2 as (PHY/AAC/01).

Cream Development and Preparation: Carbopol 980 and xanthan gum, which make up the water phase, were each carefully measured out in their own container. They used a stirrer in a water bath to gently mix demineralized water into the solution. Similarly Accurate amounts of oil phase ingredients such cetostearyl alcohol, caprylic/capric triglycerides, isopropyl myristate, glyceryl monostearate, and stearic acid were measured out in a separate vessel before being heated to 80 degrees Celsius in a water bath. There was no refrigeration involved; just room-temperature cooling. The cream's recipe included the antioxidant vitamin E and the preservative phenoxyethanol. The cream was thoroughly blended for ten minutes. From lowest to maximum concentration of emulsifiers and gelling agents, the five alternative formulae were chosen to maximise viscosity, pH, and rheological tests like emulsion strength, thixo- tropicity, and elasticity. One stable formula was optimised using the five permutations shown in **Table 2** for use between 30 and 45 degrees Celsius and between 45 and 75% relative humidity.

Table 2: Formulation of cream base with different permutation combination

	Formulations				
Ingredients (%)	F-1	F-2	F-3	F-4	F-5
Carbopol 980	0.25	0.5	0.6	0.65	0.7
Xanthan gum	0	0.1	0.2	0.25	0.3
Cetyl alcohol	1	2	2.25	2.5	2.75
Cetostearyl alcohol	0.5	1	1.25	1.5	1.75
Glyceryl monostearate	1	2	2.25	2.5	2.75
Stearic acid	3.5	4	4.25	4.5	4.75
Capryliccapric triglycerides	1	2	2.25	2.5	2.75
Isopropyl myristate	1	2	2.25	2.5	2.75
Water (demineralised)	86.28	72.93	79.23	77.63	76.03
Glycerine	3	3	3	3	3
Sodium hydroxide	0.2	0.2	0.2	0.2	0.2
Vitamin E acetate	0.3	0.3	0.3	0.3	0.3
Phenoxy ethanol	0.9	0.9	0.9	0.9	0.9
Perfume Make-soothing inde	1.0	1.0	1.0	1.0	1.0
Color-Make-1% Ponceau	0.07	0.07	0.07	0.07	0.07

Evaluating Cytotoxicity: Cytotoxic activity of the poly-herbal extract mix and cream recipe was evaluated in HDF and NIH 3T3 cells using a 3-(4, 5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (Sigma) MTT assay. For 24 hours, 1 x 104 cells/mL of both cell types were cultivated in 96-well plates with media supplemented with varying amounts of poly-herbal extract and the final cream formulation (1000-15.625 g/mL). Ten microliters of MTT (5 mg/mL) were applied to each well, and the cells were then incubated at 37 degrees Celsius for four hours. After removing the supernatant, 100 L of dimethyl sulfoxide (Merck) was added to each well in order to dissolve the Formazan product. The absorbance was measured spectrophotometrically at 540 nm with an ELISA reader (Synergy HT multi-detection micro plate reader). Non-toxic concentrations (CTC50 values) were determined for both the extract and the cream formulation, and they were used in further experiments.

ELISA for Measurement of Collagen: Quantitative measurements of collagen-I (Blue Gene Biotech, China) in human dermal fibroblast cell supernatants were accomplished using an enzyme-linked immunosorbent assay (ELISA) (HDF). HDF cells were treated with the test substances, then incubated for 96 hours at 37 degrees Celsius. Collecting the cells' supernatant for ELISA was the next step. The ELISA was performed as directed by the kit's manual. A. squamosa (ASW) and M. koengii (MKW) and R. damascena (RD) water extracts were tested separately (RDW),

We measured collagen using a combination of C. paniculatum (CPW) and three different extract blends (CB 01, CB 02, and CB 03). The ultimate concoction (PHY/AAC Collagen concentration was measured in /02) and CB 03 (the best poly-herbal extract mix). The 450 nm wavelength was used to determine the color's relationship to collagen content.

Polymerase Chain Reaction, Partially Quantitative: For RT PCR, we decided on the

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extract mix (CB 03) found in the final formulation (PHY/AAC/02) because to its high collagen-I content. Using the TRI reagent, total RNA was isolated from the cells (Sigma). Denaturing 1% agarose gel was used to determine how much RNA was extracted. For cDNA synthesis, Reverse transcriptase was employed with total RNA and OligodT. Utilizing collagen type I-specific primers, polymerase chain reaction (PCR) reactions were performed with the following operating conditions: initial denaturation at 95 °C for 5 minutes, followed by 40 cycles, with each cycle consisting of initial denaturation for 60 seconds at 95 °C, annealing for 60 seconds at 45 °C, and elongation for 90 seconds at 72 °C. The last 10 minutes of the extension were performed at 72 degrees Celsius. Ethidium bromide-stained 1.5% Agarose gels were used to record the final PCR products after they had been resolved. Comparison of the band intensity of the amplified cDNA with a conventional molecular weight marker (1 Kb ladder) allowed us to determine its molecular weight. Image J was used for the densitometric analysis of the amplification of genes.

The Rheological Behavior: Rheology is the study of how a product flows, and it provides valuable insight into the qualities of the product. It was found by analysing the flow curve, amplitude sweep, frequency sweep, and thixotropic qualities that the product's behaviour may be understood in terms of flow properties, such as the recovery of the strain rate, among other things. Final cream formulas with and without active (PHY/AAC/01 and PHY/AAC/02) as well as a market sample (PHY/AAC/03) were subjected to comparative rheological tests utilising a Modular Compact Rheometer (MCR-302, Anton Paar).

Studies of Stability: Thermo lab stability chambers were used to test the extracts (CB01, CB02, and CB03), cream formula (PHY/AAC/01), cream formula (PHY/AAC/02), and market sample (PHY/AAC/03) for shelf life stability (Thane, India). Final product stability was evaluated for 6 months at both real-world circumstances (at 30 degrees Celsius and 65 percent relative humidity) and accelerated conditions (at 45 degrees Celsius and 75 percent relative humidity), as required by ICH recommendations.

Consistency forecasting relies heavily on variables like viscosity and pH. Using a viscometer (with a 1 rpm T-96 spindle), we were able to get an approximation of the viscosity (Model-DV2T, Brooke field). PHY/AAC/01 is a cream formula without the active blend; PHY/AAC/02 is a cream formula with CB 03 active blend; and PHY/AAC/03 is a market sample. Initial, first, second, third, and sixth month pH were measured using the pH metre (Model number-F-71, Horiba scientific) in both real-time (30 °C and 65% RH) and accelerated conditions (45 °C and 75% RH).

RESULTS AND DISCUSSION:

In this case, we extracted the active compounds from R. damascena, C. paniculatum, M. koengii, and A. Squamosa. Collagen concentrations in several plant extracts were measured. In comparison to the reference ascorbic acid concentration of 16.53 0.064ng/ml, the collagen concentrations were 15.23ng/ml, 11.79ng/ml, 10.92ng/ml, and 6.78ng/ml, respectively. The combination was added to a cream base in three distinct ratios.

Assessment of Polyherbal Formulations for Cytotoxicity: Before adding the poly-herbal formula into the cream base, it was subjected to this assay to determine its safety. The MTT test was used to assess the cytotoxic activity of poly-herbal active blends (CB01), (CB02), and (CB03). All doses examined demonstrated that NIH 3T3 cell viability was unaffected by a little increase in the cytotoxic concentration up to 1000 g/mL. As can be shown in Fig.1, the LC50 values for the maximum concentration of (CB01), (CB02), and (CB03) tested (28.5± 0.075), 32.57± 0.32, and 22.51± 0.03, respectively, were all hazardous. Among the three extract blends, CB 03 performed the best in terms of cytotoxicity (22.5 ±0.036% at 1000 g/mL), hence it was selected for further investigation. In the case of CB 03, the p value was found to be 0.00001, which is statistically significant at the p0.05 level. The most aesthetically pleasing and least dangerous cream formulations containing (CB 03) had cytotoxic activity of 22.5± 0.0361% at 1000 g/ml. The Cream (PHY/AAC/02) formula was updated to include it.

Measurement of Collagen Content in Separate Extracts and a Polyherbal Blend: Collagen content was evaluated using water extracts of A. squamosa (ASW), M. koengii (MKW), R. damascene (RDW), C. paniculatum (CPW), and poly-herbal blend-(CB 01), (CB 02), and (CB 03). At 0.1mg/ml, cells exposed to the usual dose of ascorbic acid (100g/ml) produced 16.53 0.064 ng/ml. In Fig. 2, sample (CB 03) with the highest collagen concentration (18.65± 0.061; p 0.05) ng/ml of Collagen -1 was used for further research.

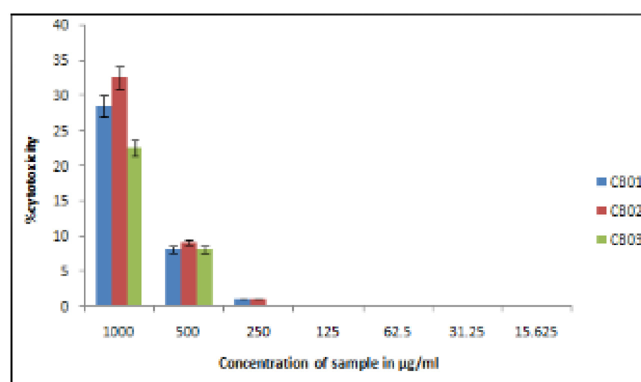

Figure 1: Assessment Of Cytotoxic Activity Of Cb 01, Cb 02 And Cb 03 Using Nih 3T3 Cell Lines Data Are Represented As Mean ± Sem (P ≤0.05) Vs Control)

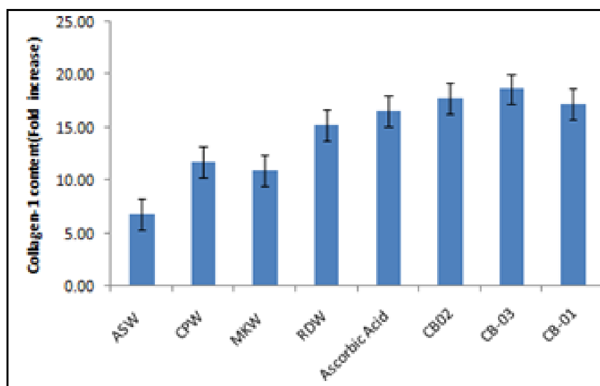

Figure 2: Estimation Of Collagen-I In Individual Extracts And Polyherbal Blends In Hdf Cells By Elisa Kit Method, Data Are Represented As Mean ± Sem (P ≤0.05) Vs Control)

Evaluation of Cream Formulation for Cytotoxicity: MTT assays were performed on cream formulations without active blend (PHY/AAC/01), cream formulations including poly-herbal blend (CB 03) (PHY/AAC/02), and a market sample (PHY/AAC/03). The outcomes are depicted in Fig. Results from Experiment 3 demonstrated that the viability of NIH 3T3 cells was unaffected by doses up to 1000 g/mL. At 1000 g/mL for 24 hours, the cyto-toxicity of (PHY/AAC/02) was 42.7± 0.18, and the nontoxic concentration was taken for further investigation. Before testing on humans, this experiment was carried out to ensure the formula was safe.

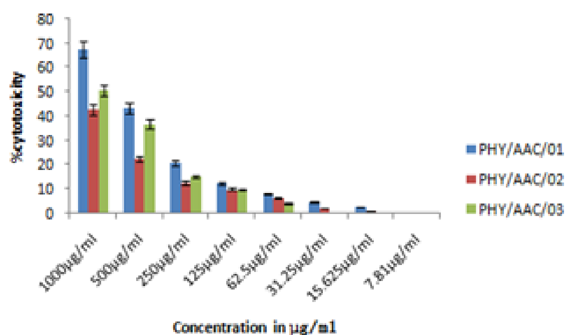

Figure 3: Assessment Of Cytotoxic Activity Of Phy/Aac/01 (Placebo), Phy/Aac/02 (In-House Sample) And Phy /Aac/03 (Market Sample) Using Nih 3T3 Cell Lines. Data Are Represented As Mean ± Sem (P ≤ 0.05) Vs Control)

Collagen-1 ELISA for Cream Formulation and Collagen Gene I Expression Study: As can be shown in Fig. 4, the Collagen-I content of the placebo cream formulation (PHY/AAC/01; 6.94 1.65 ng/mL) was significantly lower than that of the cream with the poly herbal extract blend (CB 03) (PHY/AAC/02; 17.53± 0.011 ng/mL), and the market sample (PHY/AAC/03; 11.02±0.015 ng/mL). The collagen-I content of (PHY/AAC/02) was 17.53 ±0.011 ng/mL, which is somewhat higher than the standard value of 16.53±0.064 ng/mL for Ascorbic acid (100 g/mL). With a significance level of P≤ 0.05, the finding was confirmed to be substantive.

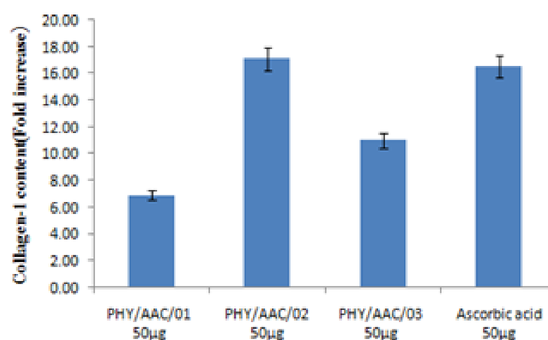

Figure 4: Collagen Content Determination In Cream Formulations Compared To Standard Data Are Represented As Mean ± Sem (P ≤ 0.05) Vs Control)

Test for Thixotropy: Samples (PHY/AAC/01, PHY/AAC/02, and PHY/AAC/03 standard [market sample]) were analysed in a shear rate ramp from 2 to 100 1/s for 165 seconds, representing the first, second, and third intervals of the thixotopic test. In the second period, the samples were analysed for 65 seconds at a constant shear rate of 100 1/s. For the third time period, the samples were sheared at a rate that decreased from 100 to 2 1/s. Thixotropic index [Pa.S] values of 265.9, 79.426 and 115.68 were determined by analysing the cream samples for structural regeneration (area under the curve). Figure displays that Cream (PHY/AAC/01) and (PHY/AAC/02) have more viscoelasticity and emulsion strength than Cream (PHY/AAC/03).5.

Stability Study: Cream formulas (PHY/AAC/01) without active blend and (PHY/AAC/02) with active blend both use the same base, which has been subjected to a temperature-dependent stability study. This ensures that the product's internal structure and emulsion structure will remain stable at 30 °C and 45 °C for a minimum of three months. Figure compares the viscosity measurements taken under normal and accelerated settings. Cream formulation (PHY/AAC/02) containing Polyherbal extract blend (CB 03) is much more stable than market sample (PHY/AAC/03) (p>0.05) during a 6-month period (6, 7). The measured viscosity was between 2,000 and 3,000 centipoise (cps) and 1,400 to 1,417 (cps) (milliseconds). A statistically significant result was discovered for the experimental condition (PHY/AAC/02), with a p value of less than 0.00001. Polyherbal blend (CB 03) comprising cream formulation (PHY/AAC/02) was discovered to be much stable at real time and accelerated condition pH and under accelerated conditions over a period of 6 months compared to a market sample (PHY/AAC/03) (Figure 1). 8 and 9. With a p value of 0.000982, the result was statistically significant at

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the 5% level, while the p value for the market sample was P >0.05, meaning that no statistically significant influence was identified.

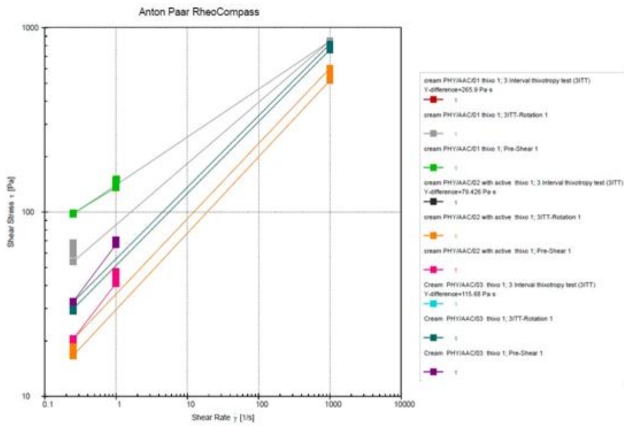

Figure 5: Thixotropic Results For Cream Base Phyaac/01 (Placebo), Phy/Aac/02 (With Active) And Phy/Aac/03(Market Sample)

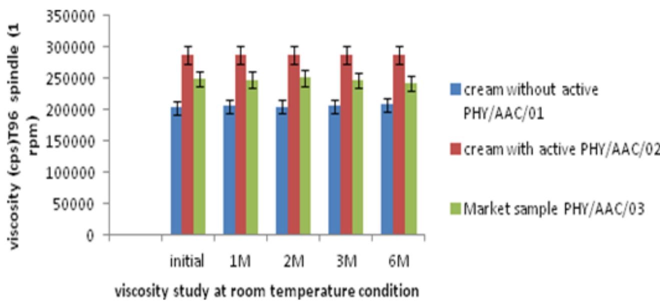

Figure 6: Viscosity Determination At Real Time Conditions (30 °C And 65% Rh) Viscosity Determination For Cream Base(Placebo), With Active, And Market Sample (45 °C And 75%Rh) Data Expressed As Mean ± Sem n=3(p≤0.005)

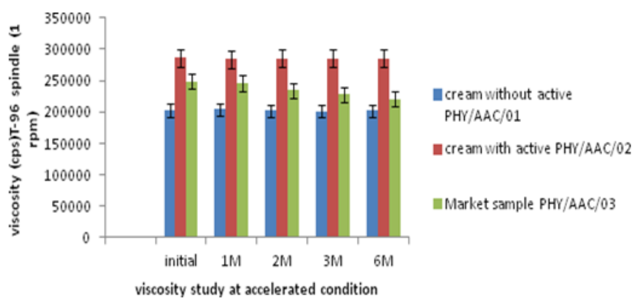

Figure 7: Viscosity Determination At Accelerated Conditions For Cream Base (Placebo), With Active, And Market Sample (45 °C And 75% Rh) Datavalues Expressed As Mean ± Sem n=3(p≤0.005)

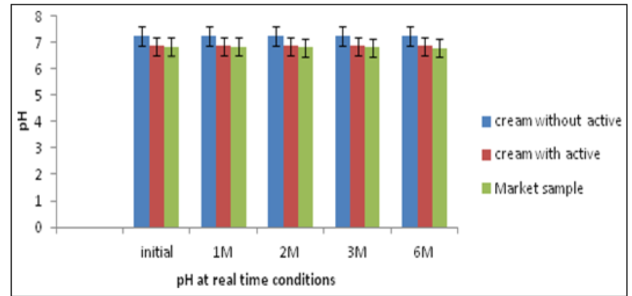

Figure 8: Ph Determination At Real-Time Stability Conditions (30 °C And 65% Rh) Datavalues Expressed As Mean ± Sem n=3(p≤0.005)

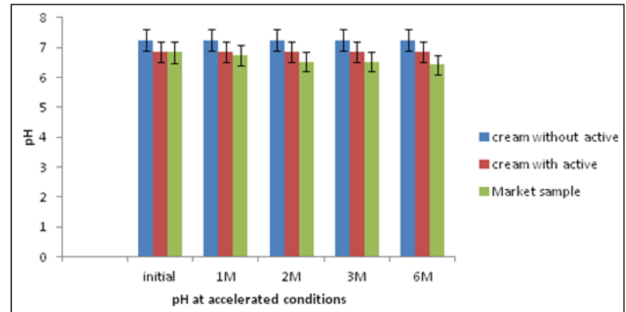

Figure 9: Ph Determination At Accelerated Stability Conditions (45 °C And 75% Rh). Data Expressed As Mean ± Sem n=3(p≤0.005)

CONCLUSION

Proper polymers and emulsifying agents were used in the development and optimization of the polyherbal cream. The combination of extracts (from M. koengii, C. paniculatum, R. damascena, and A. squamosa) helps to prevent skin dryness and revitalises the skin. The results, in both real-time and accelerated settings, demonstrated that the emulsion stability of (PHY/AAC/02) was superior. pH values varied between 5.910.16 and 6.140.01 across the temperature spectrum. The range of viscosity (in cps) was determined to be between 2.03 and 1140 17 cps and 2.87 and 687.75 cps. Upon examination of the cream's stability under accelerated stability circumstances, it was discovered to be robust. At 24 hours, the CTC50 of the combined cream base (with and without extract blend) was greater than 1000 g/mL. Measurements of thixotropy, amplitude, amplitude sweep, and flow curves revealed that the cream formulation possesses remarkable emulsion strength, viscoelasticity, shear thinning characteristic, and spreadability.

Collagen quantification data showed that HDF cells treated with cream containing polyherbal extract (CB 03; PHY/AAC/02) had increased expression of the collagen gene I. For the first time, this article details the in vitro effects of a polyherbal extract blend made from four plants native to India on human skin cells. Specifically, the polyherbal extract blend (CB 03) used in the cream formulation (PHY/AAC/02) protects the skin and stimulates the production of

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collagen and other structural proteins in the skin. When applied topically, the cream formulation (PHY/AAC/02) containing the active blend (CB03) protects the skin from injury by increasing collagen production. Increased collagen content in skin after using the cream regularly demonstrates the production of collagen matrix and repairs damaged skin for a variety of clinical situations, primarily to improve skin vitality and rejuvenation**.**

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