

Journal of Molecular Science

www.jmolecularsci.com

ISSN:1000-9035

Development of Topically Applied Herbal Cosmetic Formulations for Age Defying Effects

Anamika Singh¹, Anurag Verma¹, Prashant Kumar^{1,2},¹Teerthanker Mahaveer College of Pharmacy, Teerthanker Mahaveer University, Moradabad-244001, Uttar Pradesh, India²SRM Modinagar College of Pharmacy, SRM Institute of Science and Technology Delhi-NCR Campus, Delhi-Meerut Road, Modinagar, Ghaziabad, Uttar Pradesh, 201204, India

Article Information

Received: 24-08-2026

Revised: 12-09-2026

Accepted: 26-09-2026

Published: 08-10-2026

Keywords

Herbal, cosmetic cream, particle size, antioxidant assays, cytotoxicity

ABSTRACT

In the present investigation an attempt has been made to prepare and evaluate *in vitro* herbal cosmetic cream for its potential antioxidant effect on skin. Three cream formulations (F1-F3) were prepared using *Withania somnifera* root powder, *Asparagus racemosus* root powder, *Glycyrrhiza glabra* root powder and *Commiphora wightii* dried resin powder as herbal constituents. Other ingredients used were *Aloe vera* gel, Xanthan gum and Virgin coconut oil. The herbal constituents were extracted in virgin coconut oil (cold maceration for 25 days) and in ethanol (95%) using soxhlet extractor, separately. These extracts were than mixed and alcohol portion was removed by heating on a magnetic stirrer at 60 degrees centigrade for 2 hours. The augmented virgin coconut oil extract was then mixed with *Aloe vera* gel (1:1) using xanthan gum as stabiliser. The prepared o/w cream formulations were evaluated for physical stability of emulsion, spreadability and particle size analysis. The optimized formulation was subjected to *in vitro* antioxidant assays (DPPH, ABTS, FRAP), *in vitro* cytotoxicity assay (MTT) and *in vitro* enzyme inhibition assays (Catalase, Hyaluronidase and Elastase). The optimized cream formulation showed negligible cytotoxicity on HaCat cell lines and good *in vitro* antioxidant activity as evidenced by above mentioned antioxidants assay. From the results obtained, it is postulated that the prepared cream formulation showed sufficient promise to be further develop and scale up as promising cosmetic formulation.

©2026 The authors

This is an Open Access article distributed under the terms of the Creative Commons Attribution (CC BY NC), which permits unrestricted use, distribution, and reproduction in any medium, as long as the original authors and source are cited. No permission is required from the authors or the publishers. (<https://creativecommons.org/licenses/by-nc/4.0/>)

1. INTRODUCTION:

The word herbal cosmetic formulation refers to the topically applied cosmetic product for intended skin rejuvenating effects. These formulations are expected to be gentler on the skin and also within the reach of people who cannot afford to buy costly skin cosmetics at present flooded in the market. From the number of references, it become known to us that herbs possess a

number of skin rejuvenating agents such as antioxidants, anti-inflammatory agents, soothing agents etc [1,2]. Beside the above, from a survey report, it is known to us that the herbal cosmetics market size is expected to grow from USD 103.94 billion in 2023 to USD 145.10 billion by 2028, at a CAGR of 6.90% during the forecast period (2023-2028). The growing preference for chemical-free products and the rising popularity of environmentally-sustainable products are driving the demand for herbal cosmetics worldwide [3,4]. This prompted us to evaluate the cosmetic potential of selected herbs including *Withania somnifera* root powder, *Asparagus racemosus* root powder, *Glycyrrhiza glabra* root powder and *Commiphora wightii* dried resin powder [1,2,5,6]. These herbs are widely reported on cosmetic blogs and sites managed by cosmetic companies as wonder herbs for skin rejuvenation. In majority of cases the information about the cosmetic formulations, herbs

used and their skin rejuvenating characterization results are not completely disclosed because these are patented. In light of the above an attempt has been made to scientifically check the potential of these herbs incorporated in a cosmetic cream *in vitro*.

2. Experimental

2.1 Materials

Virgin coconut oil and *Aloe vera* gel (*Aloe barbadensis*) were procured from Patanjali Ayurveda, India. Xanthan gum and ethanol (95%) were procured from Sigma Aldrich. *Withania Somnifera* root powder, *Asparagus racemosus* root powder, *Glycyrrhiza glabra* root powder and *Commiphora wightii* dried resin powder were purchased from Patanjali Ayurveda,

India. All other chemicals used were of analytical grade (AR).

2.2 Preparation of Herb-Infused Oil by Maceration Method [7,8]

Briefly, accurately weighed amount of powdered drugs were mixed in virgin coconut oil (50g) and 50 ml ethanol (99%) separately. The oil portion was kept at room temperature (30°C) with occasional stirring for 25 days in a glass jar fitted with a lid. The ethanolic portion was extracted by soxhlet extractor at 60°C for three hours. After filtration, the volume of filtrate was noted and adjusted to 50 ml in case of oil fraction. The ethanol fraction was evaporated to 5 ml on a water bath set at 60°C (Table 1).

Table 1: Composition of herb infused oil and ethanolic fractions

Composition	Oil fraction			Herb reinforced oil- Ethanolic fraction		
	F1	F2	F3	F1	F2	F3
<i>Withania Somnifera</i> root powder (g)	1	2	3	1	2	3
<i>Asparagus racemosus</i> root powder (g)	1	2	3	1	2	3
<i>Glycyrrhiza glabra</i> root powder (g)	1	2	3	1	2	3
<i>Commiphora wightii</i> dried resin powder (g)	1	2	3	1	2	3
Virgin coconut oil (g)	50	50	50			
Ethanol (99%) (ml)	-	-	-	50	50	50
Final volume after evaporation				5	5	5

2.3 Preparation of cream formulations:

The oil phase was mixed with the ethanolic fraction and heated (60°C) to remove ethanol as completely as possible. The augmented oil extract so prepared was mixed with *Aloe vera* gel and Xanthan gum. Overall three formulations (Table 2) were prepared. The o/w

cream formulations were prepared by slowly adding the oil phase to the *Aloe vera* gel containing xanthan gum while stirring (1500 rpm) continuously at room temperature (30°C) to obtain smooth o/w emulsions [9].

Table 2: Formulation composition of herb infused cosmetic emulsions

Formulation code	Aloe vera gel (gm)*	Herb infused Virgin coconut oil fraction. (ml)	Herb infused Ethanolic fraction. (ml)	Xanthan Gum (mg)
F1	50	50	5	100
F2	50	50	5	100
F3	50	50	5	100

2.4 Physical characterization of Emulsion: [10][15]

The emulsions prepared as above were characterized for their appearance, viscosity, pH, stability, and droplet size.

2.4.1 Centrifuge Test:

10 g of each formulation was placed in centrifugal tubes and centrifuged at 3000 rpm for 30 min with a centrifuge diameter of 8.5 mm (name of the instrument).

2.4.2 Viscosity Measurement:

Viscosity measurements (Brookfield) were made at constant temperature (25°C) at 100 rev. /min. The runs were carried out 1 h and/or 24 h after the preparation.

2.4.3 pH Measurement:

Briefly, approximately 1 g of the cream formulations was dispersed in 10 mL of distilled water and the pH was measured in a digital pH meter (name of the instrument) previously calibrated at 28°C.

2.4.4 Spreadability test:

The cream formulations (2 g) were placed within a circumference of 1 cm diameter pre-marked on a glass plate (Proaño et al., 2020). Then, a second glass was placed over with a weight of 467.8 g and allowed to rest for five min.

2.4.5 Globule Size Distribution:

Globule size distribution, z-average diameter, zeta potential, and polydispersity index (PDI) of microemulsions were assessed using a nano zeta size, zeta potential, and PDI analyzer (ZS-90, Malvern Instruments, Malvern, UK) [7].

2.5 In vitro Antioxidant Assays:

2.5.1 DPPH Assay:

The free radical DPPH was used as an antioxidant with which the antioxidant activities were measured. A typical antioxidant assay is founded on the scavenging of the DPPH-free radical [11,12]. In short, 1 ml of 100 µM DPPH in methanol was added to 1 ml of the diluted

sample solution in phosphate buffer (pH 7.4) and mixed thoroughly and then, the test tubes were incubated in a dark room during 30 minutes. Each solution was picked and incubated at 37°C/30 min followed by determination of the absorbance of each at 520 nm in a double beam UV spectrophotometer. The blank readings were also recorded and the remaining DPPH free radical calculated by the use of the following formula:

$$\% \text{ Inhibition} = \frac{AbC - AbS}{AbC} \times 100$$

Where AbC is the absorbance of control and AbS is the absorbance of the sample/standard. The experiment was repeated thrice. The six points of concentration were measured. Ascorbic acid (ASC) was taken as standard [9].

2.5.2 ABTS Assay:

The antioxidant capacity was assessed using the ABTS assay, which involves the decolourization of the ABTS radical cation. The ABTS^{•+} cation radical was generated by reacting 7 mM ABTS with 2.45 mM potassium persulfate, followed by storage in the dark at room temperature for 12-16 hours. The ABTS^{•+} solution was diluted to achieve an absorbance of 0.700 at 734 nm. After adding 5 µl of plant extract to 3.995 ml of diluted ABTS^{•+} solution, absorbance was measured after 30 minutes. A solvent blank was included in each assay, and all measurements were repeated at least three times. The percent inhibition of absorbance at 734 nm was calculated [13].

2.5.3 FRAP Assay:

The Ferric Reducing Antioxidant Power (FRAP) assay evaluates the reduction of ferric ions (Fe³⁺) to ferrous ions (Fe²⁺) using a spectrophotometric method. Following Benzie and Strain's procedure, the reduction of the Fe³⁺ TPTZ complex to a blue Fe²⁺-tripyridyl triazine complex is monitored by measuring absorbance at 593 nm. The FRAP reagent is composed of acetate buffer, TPTZ in HCl, and FeCl₃.6H₂O mixed in a 10:1:1 ratio. After mixing with plant samples, the absorbance is recorded after a 30-minute incubation. A calibration curve is established by plotting absorbance against FeCl₃ concentrations, allowing the determination of FRAP based on the absorbance change [13].

2.6 In vitro enzyme inhibition assays [14,15]

2.6.1 Catalase Inhibition Assay

In catalase inhibition assay on cosmetic formulations (F, F2, F3), 5 µl of each cosmetic formulations in different concentrations were taken in phosphate buffer (pH 7.4) along with Catalase enzyme stock solution (10 unit/ml). Reaction mixture was incubated for 10 minutes. After incubation, 80 µl of the substrate (H₂O₂) was added to each reaction mixture and incubated for

5 minutes. Finally, 100 µl Ammonium Molybdate solution (32.4 mM in 25 mM H₂SO₄) was added to each well and reading was taken at 405 nm.

2.6.2 Hyaluronidase Inhibition Assay:

Sample dilutions of (0-500 µg/ml) in DMSO were prepared. Enzyme solution (50 µl) containing 10U/ml Hyaluronidase was placed in defined well of a 96-well plate. Samples (10 µl) were added, and mixture was incubated for 10 minutes. The reaction was initiated by adding 50 µl substrate (Hyaluronic Acid, 0.3 mg/ml) and mixture was further incubated for 45 minutes. Finally, after 45 minutes 100 µl Acidic Albumin solution was added and then plate was incubated at room temperature for 10 minutes and absorbance was taken at 595 nm using a micro plate reader (iMark, BioRad). IC 50 was calculated by using software Graph Pad Prism 6.

2.6.3 Elastase Inhibition Assays:

100 mM Tris-HCl buffer (pH 8.0) was used in the elastase enzyme inhibition assay. It entails mixing 1 mg/ml of elastase enzyme stock solution with 5 µl of the test sample (0-1000 µg/ml) and kept aside for 15 minutes. 10 µl of 1.0 mM N-succinyl-Ala-Ala-Ala-p-nitroanilide substrate was added to initiate the reaction, and absorbance at 405/660 nm was used to measure p-nitroaniline release. Using GraphPad Prism 6 software and non-linear regression analysis, IC₅₀ values were calculated using a positive control, 100 mM epigallocatechin-3-gallate (EGCG).

2.7 In vitro cytotoxicity assay:

The cytotoxicity of the selected emulsion formulation was evaluated using the MTT assay, which measures cell viability in HaCat cell lines [16].

2.7.1 MTT Assay:

The MTT assay was performed in quadruplets on HaCat cell line to assess the cytotoxicity of cosmetic formulation F3, containing the highest concentration of botanical extractives. HaCaT cells were cultured in a 96 well plate for 24 hours in DMEM medium with 10% FBS and 1% antibiotics at 37°C with 5% CO₂. Following treatment with various concentrations for another 24 hours, MTT solution was added, and after 2 hours of incubation, the supernatant was removed. The cell layer was dissolved in Dimethyl Sulfoxide and read at 540 nm and 660 nm using an Elisa plate reader. The IC-50 value was determined using Graph Pad Prism -6. Cell viability correlated with absorbance levels, where higher absorbance indicated more viable cells.

3. RESULTS AND DISCUSSION:

3.1 Selection of herbs:

3.1.1 Virgin coconut oil:

Coconut oil was chosen because it is a good moisturizer on any kind of skin even the dry one. The advantage of

coconut oil on the skin is similar to the advantage of mineral oil. Luckily, when using coconut oil, one does not have any negative reaction on the skin as is the case with mineral oil. Coconut oil is hence a safe option in averting skin drying and flaking. It also postpones wrinkling and skin sagging that are usual related to old age. Coconut oil is also useful in curing all types of skin issues such as psoriasis, dermatitis, eczema among other skin infections. It is due to the same reason that coconut oil is the base ingredient of other body care products like soaps, lotions, creams that are applied to the skin. The prevention of premature aging and degenerative diseases because of the well-known antioxidant properties of coconut oil also contributes to its use. [17].

3.1.2 Commiphora wightii Dried Resin Powder:

C. wightii is commonly known as ‘Indian bdellium’, or ‘Guggul’, in India. *Commiphora wightii* contains large number of phytochemical steroids, guggultetrol, lignans, amino acid, flavanoid and terpenoids. It has endowed with enormous pharmacological values due to various types of guggulsterones. The therapeutic uses include curing skin diseases, leprosy, hypertension, ulcerative pharyngitis, and urinary diseases [11,18].

3.1.3 Withania somnifera Root Powder:

Withania somnifera, known as Ashwagandha, contains more than 40 withanolides and numerous alkaloids. Modern research validates its uses, revealing anticancer, anti-inflammatory, and immunomodulatory effects. It also shows effectiveness against neurological disorders, psychological conditions, and has antimicrobial properties [12,19].

3.1.3 Asparagus racemosus Root Powder:

Asparagus racemosus, or Shatavari, rich in bioactives such as steroidal saponins and flavonoids, it possesses antimicrobial, anti-inflammatory, and antioxidant properties [6].

3.1.4 Glycyrrhiza glabra Root Powder:

Glycyrrhiza glabra roots are used to prevent and treat a number of issues, particularly skin irritation, cancer, and microbial/viral infections. Flavonoids are the most significant bioactive chemicals, accounting for the majority of biological activity. Numerous phytochemicals, such as glycyrrhizin, 18 β -glycyrrhetic acid, glabrin A and B, or isoflavones, have been found and linked to the biological activities reported, such as hepatoprotection, antioxidant, antiviral, antimicrobial, anticancer, or anti-inflammatory properties [7].

3.2 Preparation of emulsion

3.2.1 Physical characterization of emulsion formulations:

The Central Instrumentation Facility (CIF), BIT Mesra, Ranchi, India estimated particle size and zeta potential (Malvern zeta sizer MAL 500999 Version 6). These parameters affect numerous characteristics of the particulate material and are an effective measure of the quality and performance (5) and can be identified through a dynamic light scattering (DLS) device interpretation of the Z-average, polydispersity index (PDI), and distribution estimation.[9].

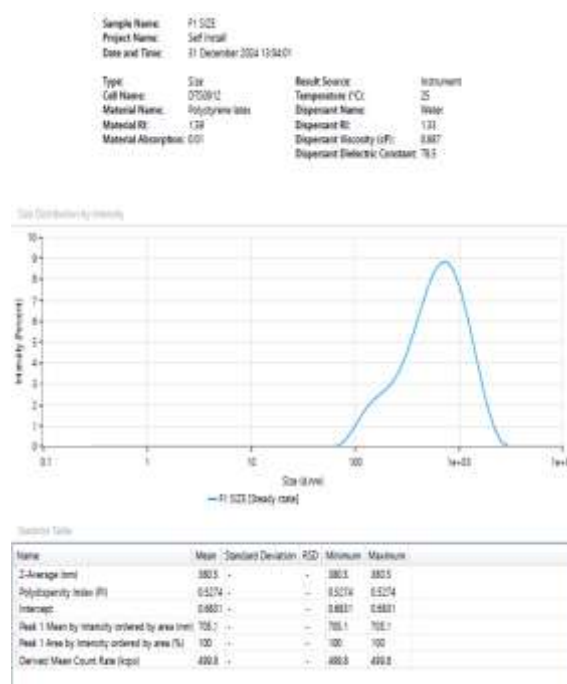


Figure 1: Globule size distribution of cosmetic emulsion formulation F1

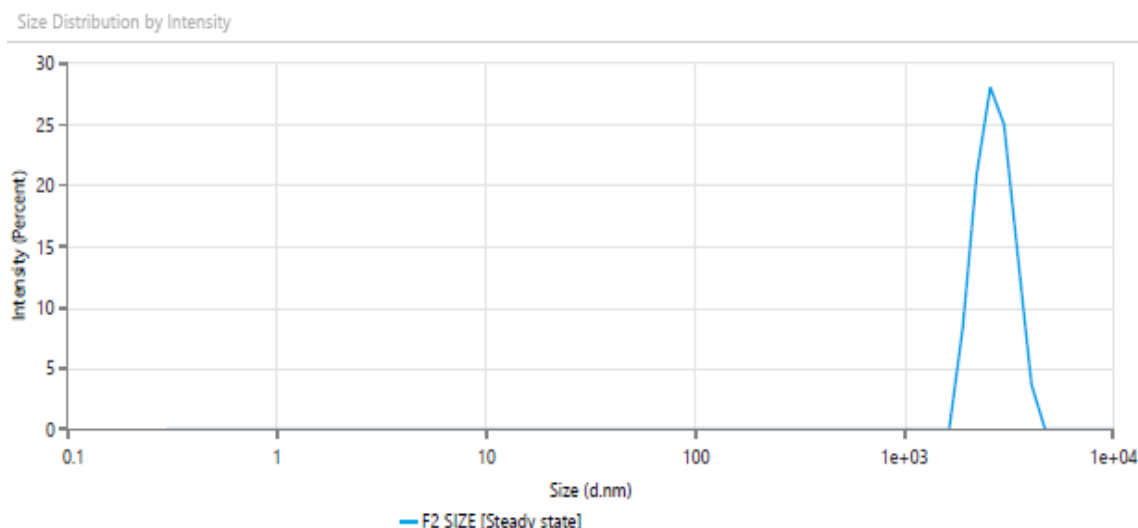
3.2.2 Polydispersity index (PDI):

Table 2 shows the PDI of different formulations. The PDI is used to measure the particle size distribution width. In the case of DLS, the optimal PDI is 0.0 (infinitely thin delta function) and the poorest PDI is 1.0. The PDI of 1 means that the sample consists of very wide size distribution, and it might have large particles or aggregates which can stabilize slowly. The values of PDI below 0.1 are monodisperse and above 0.1 are multimodal. The findings indicated that the value of PDI between all the results was. the formulations was less than 1 and ranged from 0.353 to 0.939, which shows that droplets are homogeneously distributed throughout the dispersion media.

Sample Name: F2 SIZE
 Project Name: Self Install
 Date and Time: 09 January 2025 14:44:27

Type: Size
 Cell Name: DTS0012
 Material Name: Polystyrene latex
 Material RI: 1.59
 Material Absorption: 0.01

Result Source: Instrument
 Temperature (°C): 25
 Dispersant Name: Water
 Dispersant RI: 1.33
 Dispersant Viscosity (cP): 0.887
 Dispersant Dielectric Constant: 78.5



Statistics Table

Name	Mean	Standard Deviation	RSD	Minimum	Maximum
Z-Average (nm)	2574	-	-	2574	2574
Polydispersity Index (PI)	0.1089	-	-	0.1089	0.1089
Intercept	0.8263	-	-	0.8263	0.8263
Peak 1 Mean by Intensity ordered by area (nm)	2727	-	-	2727	2727
Peak 1 Area by Intensity ordered by area (%)	100	-	-	100	100
Derived Mean Count Rate (kcps)	1670	-	-	1670	1670

Figure 2: Globule size distribution of cosmetic emulsion formulation F2

Table 3: Physical characterization of emulsion formulations

Parameters	F1	F2	F3
Globule size (nm)	705.1	2727	1639
Area (%)	100%	100%	100%
Z-Average Diameter (nm)	380.5	2754	1197
Polydispersity Index (PDI)	0.5274	0.1089	0.3214
Zeta Potential (mV)	-52.87	-40.24	-35.98
Intercept	0.6831	0.8263	0.6751
pH	6.3	6.1	5.9
Viscosity (cP)	750	1250	1280
Centrifugal Stability	Stable	Stable	Stable

3.2.3 Zeta Potential:

The zeta potential is a significant physical property of dispersed particles, aiding in the optimization of suspension and emulsion formulations. Understanding zeta potential can significantly shorten the formulation

trial process. The measured zeta potential values for emulsions ranged from -35.98 to -52.87 mV, indicating that formulations are generally considered stable if they exceed $+30$ mV or fall below -30 mV.



Figure 3: Globule size distribution of cosmetic emulsion formulation F3

3.2.4 Z- Average diameter:

The Z-average value, or "cumulants mean," represents the hydrodynamic diameter's average size and serves as an intensity mean for particle size distributions. It is the standard metric for presenting results from dynamic light scattering (DLS) measurements. In monodisperse samples, the Z-average matches the mean of the single peak in the distribution, while in polydisperse samples, it contrasts with the distribution's multiple peaks. A smaller Z-average than the peak size indicates that the cumulants fit emphasize the initial decay of the correlation function, focusing on smaller distribution parts.

3.3 *In vitro* antioxidant potential of cosmetic formulations:

Antioxidant assays are based on a concept called total antioxidant capacity (TAC). TAC is measured as the amount of free radicals quenched by a test solution used to determine the antioxidant capacity of a biological sample. According to the Scopus citation indexes, ABTS [2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) together with 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and ferric-reducing antioxidant power (FRAP) antioxidant capacity assays

are among the three most popular methods. All three are commonly accepted and routinely practiced in research laboratories throughout the world [9]. In line of this DPPH Radical Scavenging Activity Assay, ABTS radical cation-based assay and Ferric reducing antioxidant power (FRAP) assay were performed on various cosmetic emulsion formulations [9].

3.3.1 ABTS Assay:

The ABTS assay measures the ability of antioxidants to scavenge the stable radical cation ABTS^{•+}. In this assay, also known as Trolox equivalent antioxidant capacity (TEAC) assay, the green-blue stable radical cationic chromophore, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS^{•+}) is produced by oxidation, and has absorption maxima at 414, 645, 734, and 815 nm. The usually applied characteristic maxima to monitor are 414–417 nm and 730–734 nm (Table 2); however, the latter is the recommended range due to the possible interference of many samples, which can be expected at lower wavelengths, resulting in the underestimated antioxidant capacity [13]. Results were presented in the table. IC-50 was calculated using Software Graph Pad Prism 6.

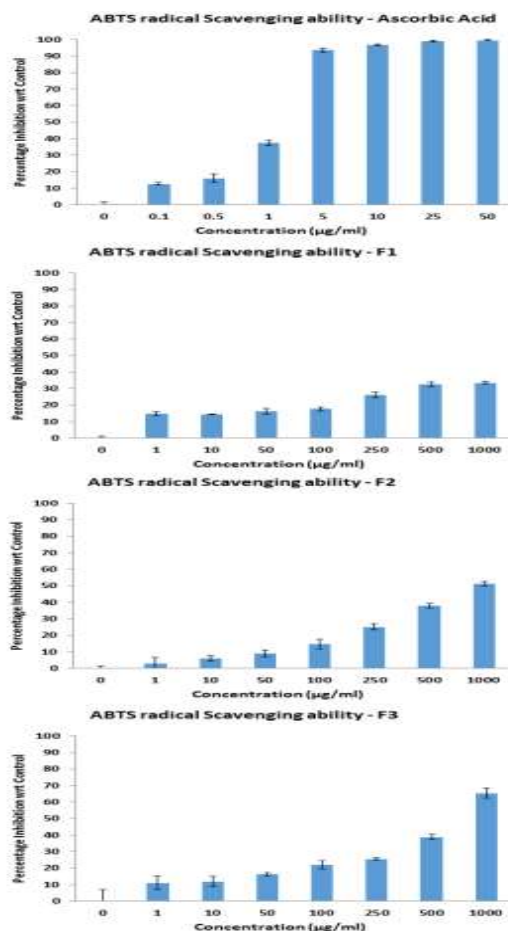


Figure 4: ABTS radical Scavenging activity of cosmetic formulations

Table 4: *In vitro* antioxidant potential of cosmetic formulations

Assay	Parameter	Standard (%)	F1 (%)	F2 (%)	F3 (%)
DPPH Assay	% Radical Scavenging Activity	94.63±2.09	-9.86±4.86	13.18±5.79	47.5±4.93
	IC ₅₀ Value (µg/mL)	6.445			1094
ABTS Assay	% Inhibition	99.64±0.73	33.50±1.72	51.19±2.35	65.30±6.05
	IC ₅₀ Value (µg/mL)	1.306	26817	970.3	700.3
FRAP Assay	FRAP Value (µmol Fe ²⁺ /mL)	141.6±16.6	51.39±6.99	84.91±14.01	100.72±5.69
	IC ₅₀ Value (µg/mL)	6.445	878	521.3	268.2

3.3.2 DPPH Assay:

Another popular antioxidant assay for cosmetics is the DPPH assay. The DPPH assay, like the ABTS assay, measures the formulation's antioxidants' ability to scavenge free radicals, which can damage skin. The DPPH assay predicts the antioxidant capacity of cosmetic formulations. By measuring the ability of the formulation to scavenge free radicals, the DPPH assay provides insights into how effectively the product can counteract the harmful effects of reactive oxygen species (ROS) in the skin. ROS are generated by various factors, including UV radiation and pollution, and can contribute to skin aging and other skin concerns. The assay can also provide information about the stability of antioxidants in the formulation. This is important because some antioxidants may degrade or lose their efficacy over time, affecting the cosmetic formulations ability to provide antioxidant protection to the skin. The primary result of a DPPH assay is expressed as the percentage inhibition of DPPH

radicals. This value indicates the extent to which the antioxidants in the sample reduced the concentration of DPPH radicals. A lower percentage inhibition indicates stronger antioxidant activity [9]. It means that the antioxidants in the sample are more effective at scavenging free radicals and neutralizing oxidative stress. DPPH assay results are compared to positive control (Ascorbic Acid). If the sample's percentage inhibition and IC₅₀ value are similar to or better than those of the positive controls, it suggests that the sample has significant antioxidant potential. The DPPH assay was performed quadruplicate using the percentage inhibition values that were obtained at various concentrations in relation to the standard (table 4 & Fig. 2). The findings of the DPPH experiment showed that the positive control displayed the highest inhibition. In the case of cosmetic emulsion formulations, it was observed that the DPPH inhibition was either too weak or not observed (Table 4).

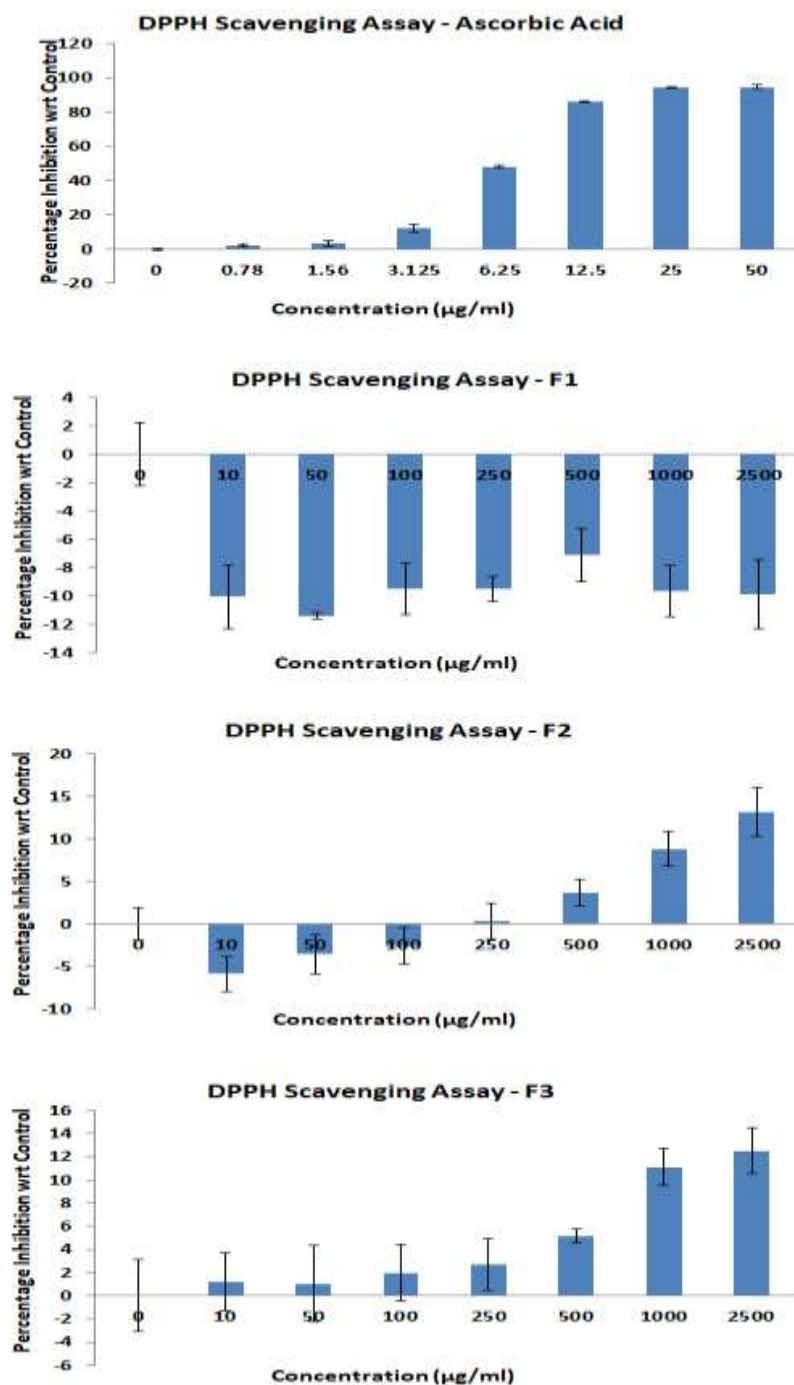


Figure 5: DPPH radical Scavenging activity of cosmetic formulations

Table 5: Percentage inhibition of the DPPH radical

Tested samples	Concentration (µg/ml)	Percentage inhibition	IC50 value
Ascorbic acid (Standard)	50	94.63±2.09	6.445
Cosmetic emulsion F1	2500	-9.86±4.86	Not observed
Cosmetic emulsion F2	2500	13.18±5.79	Not observed
Cosmetic emulsion F3	2500	12.51±3.89	Not observed
DPPH Assay on formulation F3 after 90 days of storage at room temperature (30°C)			
Ascorbic acid (Standard)	50	89.02±2.32	18.83
Cosmetic Emulsion F3	2500	47.5±4.93	1094 ± 0.035

If the DPPH assay is negative and the ABTS assay is positive, as in our case (Table 3 & 4), the herb infused cosmetic formulations in question may have distinct

antioxidant activity against DPPH and ABTS radicals. First of all, it is to be understood that DPPH, ABTS, and FRAP assays use different types of radicals as

indicators. The antioxidant's efficacy in neutralizing one type of radical may not necessarily reflect its performance with another type. The choice of assay can influence the results obtained. Further, the reaction conditions, such as pH, temperature, and incubation time, can vary between different assays. These conditions can influence the outcomes and make certain antioxidants more effective in one assay compared to another. In some cases, antioxidants may be more compatible with a specific assay system due to their chemical properties. This compatibility can lead to higher or lower antioxidant activity measurements in different assays. In an explanation, some antioxidants have varied solubility and reactivity depending on the solvent employed in the DPPH, ABTS and FRAP experiments.

In order to get more information about poor DPPH assay results, the assay on formulation F3 was performed again after storage. Formulation F3 was stored at room temperature (30°C) for 90 days in airtight containers, away from direct light. It was observed that the DPPH scavenging activity was somewhat increased significantly ($p < 0.05$). This observation could have several possible explanations.

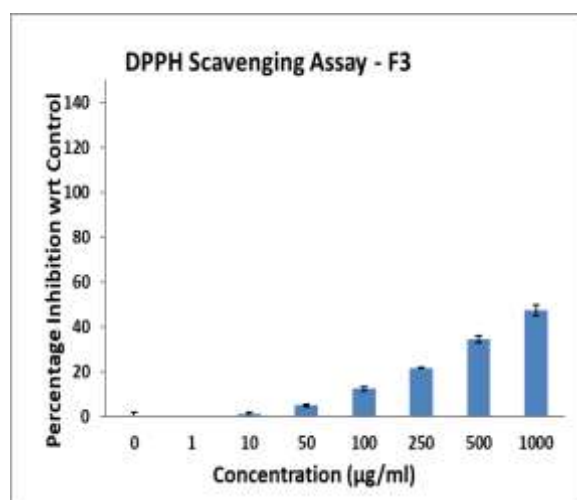


Figure 6: DPPH radical Scavenging activity of cosmetic formulations after 90 days of storage

- Interactions between different ingredients in the formulation might lead to the enhanced release or activation of antioxidants. These interactions can result in improved antioxidant performance when the cosmetic formulation is stored.
- It is also possible that the solubility of antioxidants might have improved during storage, making them more available for interactions with DPPH radicals.
- In some cases, antioxidants present in formulation F3 become more stable over time, leading to an increase in their antioxidant activity. This stabilization may be attributed to the reduced exposure to environmental factors like light, heat, or oxygen during storage.

3.3.3 FRAP Assay:

Benzie and Strain developed FRAP in 1996. The FRAP assay measures the ability of antioxidants in the sample to reduce ferric ions to ferrous ions in a redox reaction. The more antioxidants present in the sample, the higher its reducing ability, and consequently, the higher the FRAP value. Higher FRAP values suggest a higher concentration of antioxidants, which can help protect cells and tissues from oxidative damage. To interpret the FRAP results effectively, reducing power obtained from the test samples is compared to positive control (Ascorbic Acid) with established reducing power. If the samples FRAP value is similar to or higher than that of the positive control, it suggests that the sample has significant antioxidant potential [13].

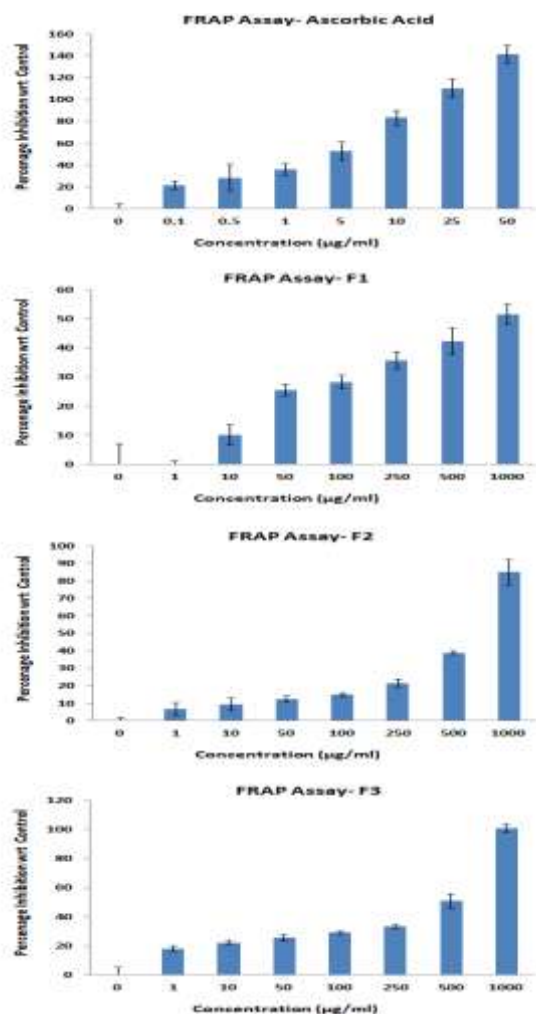


Figure 7: FRAP radical Scavenging activity of cosmetic formulations

3.3.4 ABTS assay:

The ABTS assay is an effective method to assess a herbal cosmetic product's ability to scavenge free radicals, which protects the skin from environmental stresses including UV radiation and pollution. This test helps screen products throughout development. It helps formulators choose the most effective antioxidant components by identifying formulations with increased

antioxidant activity [13].

ABTS is a substance that is both highly water soluble and chemically stable, it is well suited for usage in aqueous systems, including biological samples and aqueous extracts of plants or foods. In its oxidized state, ABTS has no discernible color; but, when it is in its reduced state, it takes on a blue-green color. It is possible to estimate the antioxidant capacity of the test compound by simply measuring the change in color using spectrophotometry, which is a straightforward process. When determining the antioxidant capacity of a substance, such as a herbal cosmetic formulation, the sample is combined with ABTS and the reaction is allowed to take place. The degree to which the herbal formulation inhibits or reduces the oxidation of ABTS is an indication of its antioxidant capacity. A higher inhibition percentage suggests that the substance possesses a more potent antioxidant activity.

In order to determine the percentage of the ABTS radical that was inhibited by the cosmetic emulsion formulation, a quadruplicate calculation was performed using the percentage inhibition values that were obtained at various concentrations in relation to the standard ascorbic acid (Fig. 1). Ascorbic acid demonstrated the highest inhibition among the studied cosmetic emulsion formulations, which suggests that ascorbic acid had powerful antioxidant activity in scavenging the ABTS radical. The findings of the ABTS experiment showed that ascorbic acid displayed the highest inhibition. In the case of cosmetic emulsion formulations, it was discovered that the antioxidant activities of the herbs rose in proportion to their concentration in the formulations, as shown by the percentage of inhibition (Table 3). This was the case even when the concentration of the herbs did not change.

Table 6: Percentage inhibition of the ABTS radical

Tested samples	Concentration (µg/ml)	Percentage inhibition	IC50 value
Ascorbic acid (Standard)	50	99.64±0.73	1.306
Cosmetic emulsion F1	1000	33.50±1.72	26817
Cosmetic emulsion F2	1000	51.19±2.35	970.3
Cosmetic emulsion F3	1000	65.30±6.05	700.3

The IC₅₀ value is a measurement of how efficient an antioxidant is at lowering the level of oxidation of ABTS that is caused by a free radical. The IC₅₀ number is the concentration of a drug that must be present in order to block the action of a radical by fifty percent. Because a lower IC₅₀ value signifies a lower concentration of the substance that is required to efficiently scavenge the free radicals, a higher antioxidant activity is indicated when this value is lower.

In contrast, an IC₅₀ value that is greater than zero

indicates that the antioxidant capacity of the molecule is lower. This is because a higher concentration of the chemical is required to achieve the same amount of inhibition. The value of IC₅₀ that is acquired from an ABTS assay can be used to compare and rank the antioxidant capacity of various compounds, as well as to evaluate the efficacy of various formulations or concentrations of a single drug. It gives a quantitative measurement of the compound's ability to neutralize free radicals and protect against oxidative stress, which is related with a variety of diseases and the aging process in general.

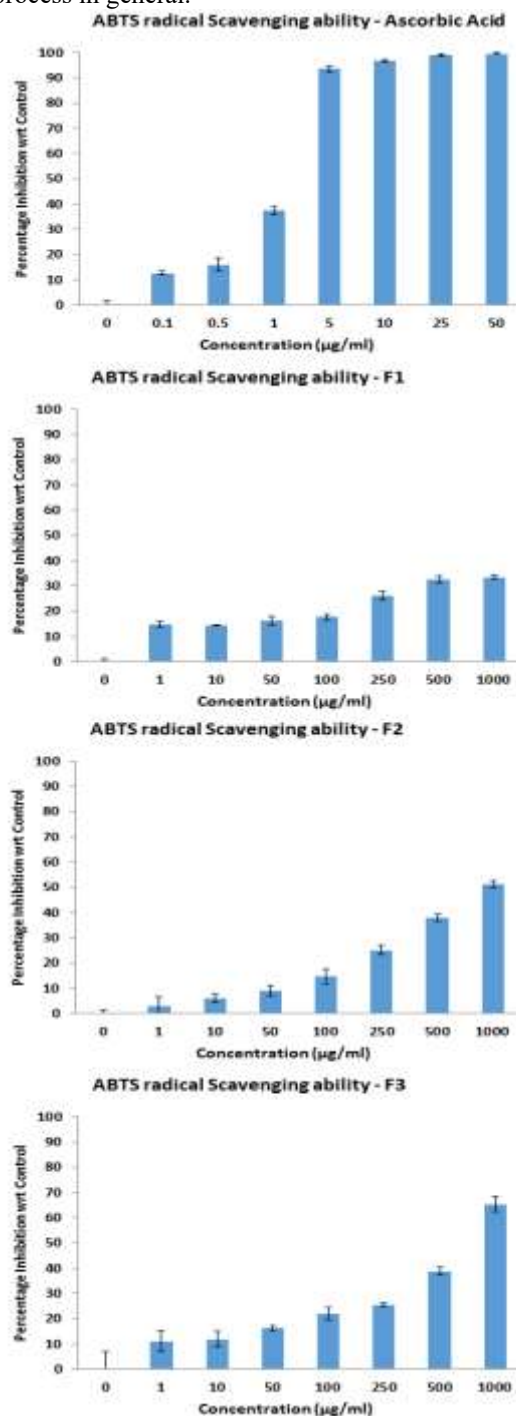


Figure 8: ABTS Radical Scavenging activity of cosmetic formulations

3.4 In vitro enzyme inhibition assays:

3.4.1 Catalase inhibition assay:

Catalase is an enzyme that plays a crucial role in protecting the skin from damage caused by reactive oxygen species (ROS), particularly hydrogen peroxide. Skin aging is often accelerated by oxidative stress, which leads to the formation of ROS. Catalase helps in neutralizing hydrogen peroxide, a common ROS. By inhibiting catalase and measuring the effect, you can assess the ability of a cosmetic product to neutralize ROS and protect the skin from oxidative damage. Catalase inhibition caused by a cosmetic formulation can have negative implications for skin health, as catalase is an essential enzyme responsible for neutralizing hydrogen peroxide and preventing oxidative damage. Inhibition of catalase can lead to the accumulation of hydrogen peroxide, a reactive oxygen species (ROS). Excessive ROS in the skin can cause oxidative stress, which damages skin cells, proteins, and DNA. This oxidative stress can contribute to premature aging. Furthermore, inhibited catalase activity can leave the skin less capable of dealing with this UV-induced oxidative stress, potentially increasing the risk of sunburn. Understanding the inhibition of catalase by cosmetic products is also crucial from a safety perspective [15,20].

In catalase inhibition assay on cosmetic formulations (F, F2, F3), 5 μ l of each cosmetic formulations in different concentrations were taken in phosphate buffer (pH 7.4) along with Catalase enzyme stock solution (10 unit/ml). Reaction mixture was incubated for 10 minutes. After incubation, 80 μ l of the substrate (H₂O₂) was added to each reaction mixture and incubated for 5 minutes. Finally, 100 μ l Ammonium Molybdate solution (32.4mM in 25mM H₂SO₄) was added to each well and reading was taken at 405 nm.

Table 7: Catalase inhibition assay of cosmetic formulations

Tested samples	Concentration (μg/ml)	Percentage inhibition	IC50 value
Sodium Azide (Standard)	10	91.49±5.48	2.705
Cosmetic emulsion F1	1000	34.36±2.37	113355
Cosmetic emulsion F2	1000	62.35±17.74	815.2
Cosmetic emulsion F3	1000	66.89±4.97	474.5

Sodium azide (NaN₃) is a well-known inhibitor of the catalase enzyme. Catalase is responsible for breaking down hydrogen peroxide (H₂O₂) into water and oxygen. Sodium azide interferes with this process by binding to the active site of the catalase enzyme, effectively inhibiting its catalytic activity. When sodium azide is used to inhibit catalase, it can lead to or maximal inhibition of the enzyme's activity, in our case it is 91.49% at 10 μ g/ml concentration (Table 6). Cosmetic formulations (F1, F2, and F3) show very low catalase inhibition compared to sodium azide.

A high IC₅₀ (half-maximal inhibitory concentration) value (Table 6), compared to positive control, for catalase inhibition may indicate that the cosmetic formulation is less likely to significantly affect catalase activity. While catalase is an important enzyme that helps protect the skin from oxidative stress the relationship between catalase inhibition and skin toxicity is more complex. The extent of catalase inhibition and the concentration of the inhibiting substance in the cosmetic formulation are important factors. A slight reduction in catalase activity may not lead to skin toxicity, while a significant inhibition could be more concerning. Since ROS and oxidative stress are known contributors to skin aging, high values of catalase inhibition may suggest a potential acceleration of premature aging, including the formation of wrinkles, fine lines, and age spots.

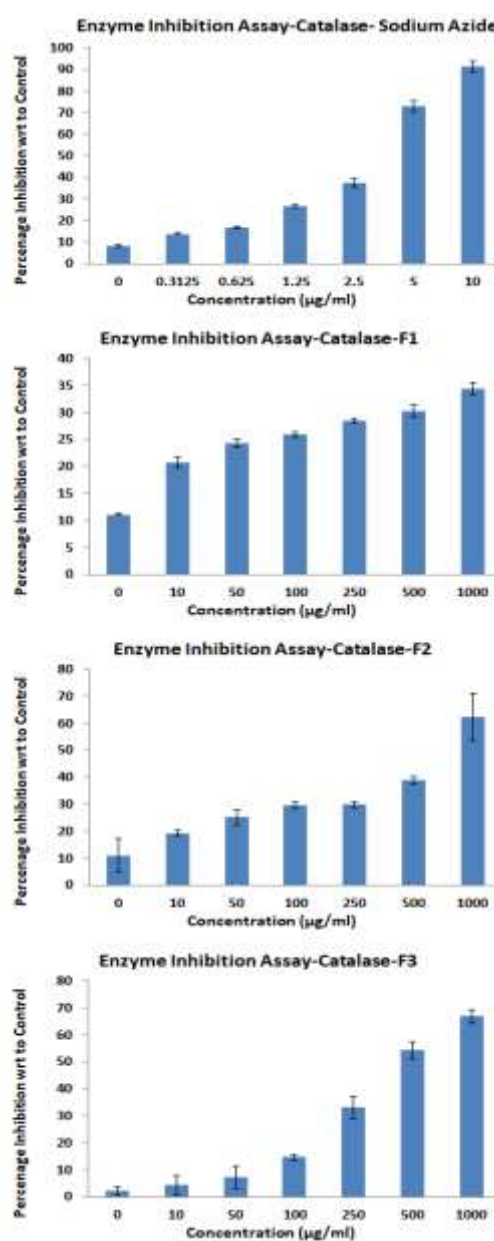


Figure 9: Catalase Inhibition Assay on cosmetic formulations

Table 8: Combined results of in vitro enzyme Inhibition Assays on cosmetic formulations

Assay	Parameter	F1 (%)	F2 (%)	F3 (%)
Hyaluronidase Inhibition Assay	% Inhibition	93.25±11.31	94.20±18.02	119.48±16.08
	IC ₅₀ Value (µg/mL)	40.12	15.35	4.58
Catalase Inhibition Assay	% Inhibition	34.36±2.36	62.35±17.74	66.89±4.97
	IC ₅₀ Value (µg/mL)	113355	815.2	474.5
Collagenase Inhibition Assay	% Inhibition			
	IC ₅₀ Value (µg/mL)			
M Elastase Inhibition Assay	% Inhibition	56.42±2.57	62.22±6.33	172.56±4.44
	IC ₅₀ Value (µg/mL)	853.9	225.8	130.9

3.4.2 Inhibition of hyaluronidase:

Hyaluronidase is not an MMP (Matrix Metalloproteinase). It is an enzyme that specifically targets hyaluronic acid, which is a major component of the extracellular matrix in connective tissues. Hyaluronidase cleaves hyaluronic acid, breaking it down into smaller fragments. By inhibiting hyaluronidase, the moisture-retaining properties of hyaluronic acid are preserved, leading to improved skin hydration and a more youthful appearance [15,20].

F3) to inhibit the activity of the elastase enzyme, which is responsible for the degradation of skin elasticity. The results in terms of IC₅₀ values indicated that:

- I. Formulation F3 demonstrated the most potent inhibitory activity with IC₅₀ value 85.39±4.36 µg/ml.
- II. Formulation F2 demonstrated IC₅₀ value 225.8±12.66 µg/ml.
- III. Formulation F1 demonstrated the IC₅₀ Value 130±6.69 µg/ml.

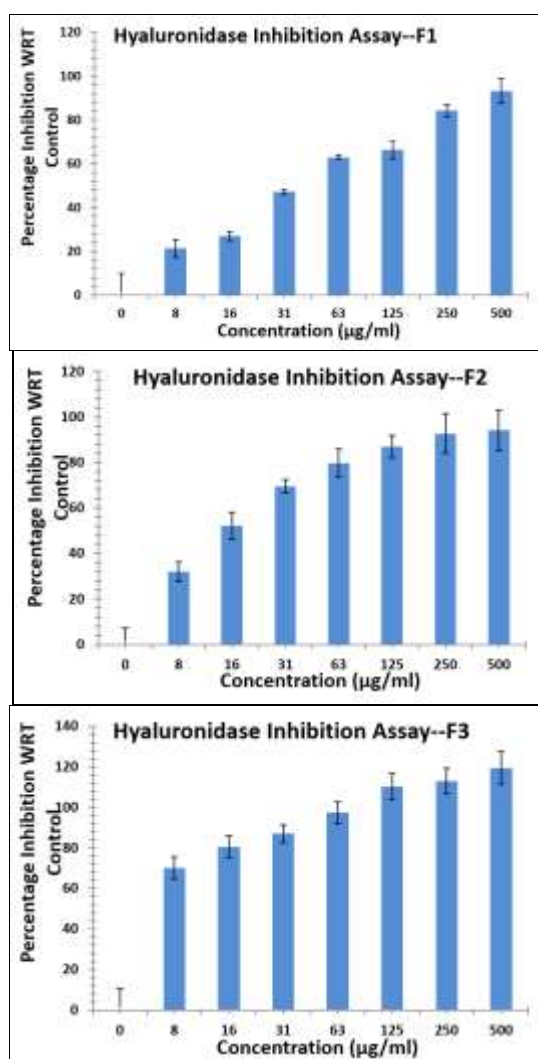


Figure 10: Hyaluronidase Inhibition Assay on cosmetic formulations

3.4.3 In vitro Elastase inhibition assay:

The elastase enzyme inhibition assay was carried out to determine the potential of the herbal formulations (F1-

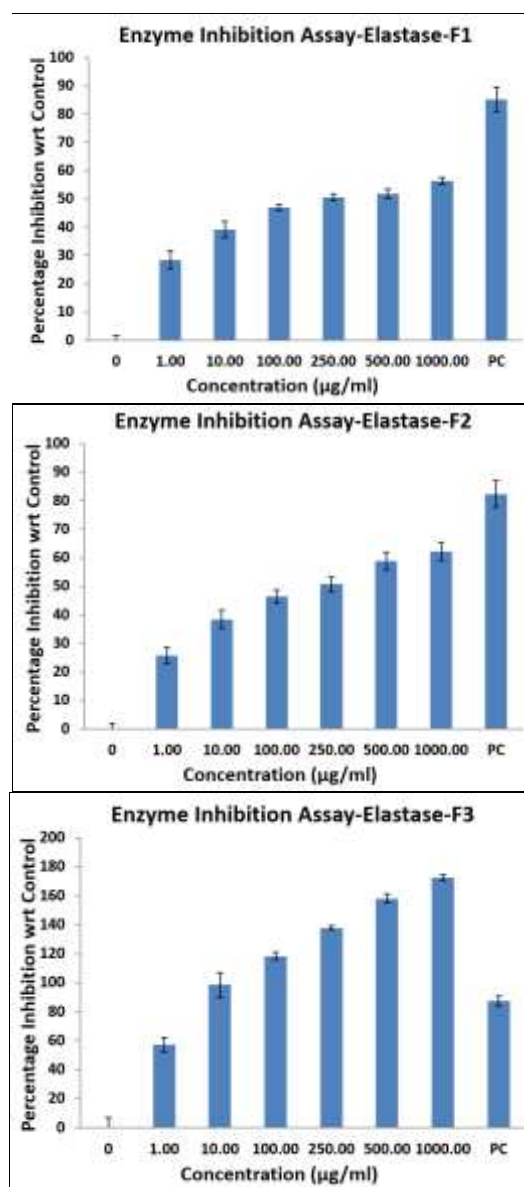


Figure 11: Elastase Inhibition Assay on cosmetic formulations

3.4.4 MTT Assay:

The MTT assay was carried out in quadruplet using HaCaT cell line. Cytotoxicity of cosmetic formulation F3 (highest botanical extractive concentration) on HaCaT (Procured from NCCS Pune- Immortal keratinocyte cell line from adult human skin) cell line was determined by MTT Assay. The cells (10000 cells/well) were cultured in 96 well plate for 24 h in DMEM medium ((Dulbecco's Modified Eagle Medium-AT149-1L) supplemented with 10% FBS (Fetal Bovine Serum - HIMEDIA-RM 10432) and 1% antibiotic solution at 37°C with 5% CO₂. Next day cells were treated from different concentrations. After incubation for 24 hours, MTT Solution was added to cell culture and further incubated for 2 h. At the end of the experiment, culture supernatant was removed and cell layer matrix was dissolved in 100 µl Dimethyl Sulfoxide and read in an Elisa plate reader (iMark, BioRad, USA) at 540 nm and 660 nm. IC-50 was calculated by using software Graph Pad Prism -6. Images were captured under inverted microscope (Olympus ek2) using Camera (AmScope digital camera 10 MP Aptima CMOS). A higher absorbance indicates more viable cells, while a lower absorbance suggests a decrease in cell viability [16].

HaCaT is a well-established human keratinocyte cell line that is commonly used for studying the effects of substances, including ethyl alcohol, on the skin. HaCaT cells are derived from human skin and, therefore, closely mimic the characteristics of human keratinocytes. This makes them a relevant model for studying the effects of substances on human skin. HaCaT cells can be used in a wide range of in vitro experiments, including toxicity assays, irritation studies, and studies on the impact of substances on skin barrier integrity [21,22].

From the results, it was evident that Very less Cytotoxic Effects against HaCaT cell line was observed in the sample F3 (IC_{50} = above dose limit i.e. 1513 ± 0.040 µg/ml).

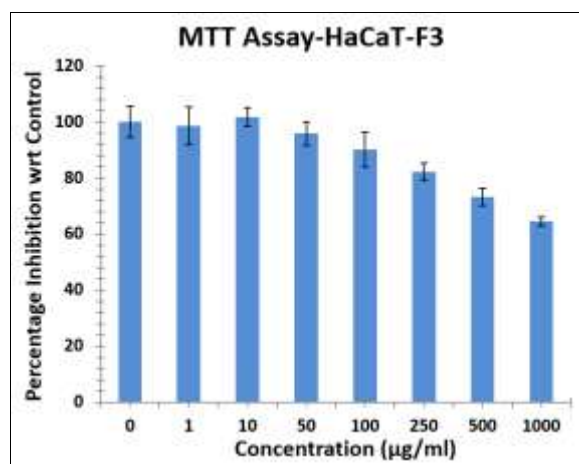


Figure 12: MTT assay on Formulation F3

4. DISCUSSION CONCLUSION:

The findings from the presented study revealed that all herbal o/w emulsion formulations maintain the desired physical characteristics for topical application. Results from in vitro antioxidant assays and enzyme inhibition assays revealed that formulation F3 was superior to the other formulations. High IC₅₀ value in case of catalase inhibition indicate that cosmetic formulations are less likely to affect the catalase activity. Formulation F3 demonstrated best in vitro antioxidant activities as evident by comparatively low IC₅₀ values obtained during in vitro antioxidant assays and enzyme inhibition studies. Cytotoxicity assay carried out on HaCaT cell lines revealed that formulation F3 was non-cytotoxic. From the study it may be concluded that herbal cosmetic formulation F3 has potential to be scale up as low cost safe and effective cosmetic product.

AUTHORS' CONTRIBUTIONS

Anamika Singh: Conceptualization, Methodology, Investigation, Data curation, Writing – original draft.

Anurag Verma: Supervision, Validation, Writing – review & editing, Project administration.

Prashant Kumar: Methodology, Formal analysis, Writing – review & editing.

Funding

The author(s) reported there is no funding associated with the work featured in this article.

CONFLICT OF INTEREST

The authors confirm that they have no financial or other conflicts of interest.

ACKNOWLEDGEMENTS

The authors would like to thank the College of Pharmacy and Teerthanker Mahaveer University in Moradabad, Uttar Pradesh, India, for their supportive research environment, academic resource access, and institutional assistance, all of which enabled this study.

ORCID

Anamika Singh:0009-0002-2670-181X

Anurag Verma: 0000-0002-2460-9967

Prashant Kumar: 0000-0001-9183-0875

REFERENCES

1. Nasralla SM. Herbal cosmetics products and formulations on the market, from past to present: an opinion. *Bull Pharm Med Res* 2023;2:9–11. <https://doi.org/10.58398/0005.000008>.
2. Suvedi D, Kumar A, Kalia S, Kumar A, Koul S, James A, Kumar D, Nagraik R, Gulilat H. Aging and Herbal Interventions: Mechanistic Insights and Therapeutic Potential. *J Cosmet Dermatol* 2025;24. <https://doi.org/10.1111/JOCD.70335>.
3. Herbal Beauty Product Market | Global Market Analysis Report - 2035 n.d. <https://www.futuremarketinsights.com/reports/herbal-beauty-products-market> (accessed January 5, 2026).
4. Herbal Beauty Products Market Share, Size, Trends 2025-2035 n.d. <https://www.metatechinsights.com/industry-insights/herbal-beauty-products-market-1961> (accessed January 5, 2026).

5. Kumar P, Verma A, Ashique S, Bhowmick M, Mohanto S, Singh A, Gupta M, Gupta A, Haider T. Unlocking the role of herbal cosmeceutical in anti-ageing and skin ageing associated diseases. *Cutan Ocul Toxicol* 2024;43:211–26. <https://doi.org/10.1080/15569527.2024.2380326>.
6. Banerjee P, Das A, Kumar P, Chakraborty S, Jalan K. An Updated Insight on the Chemistry, Ethnobotany, and Health Benefits of *Asparagus racemosus* (Shatavari): With a Special Emphasis on Shatavarin IV. *Journal of the American Nutrition Association* 2025;44:681–92. <https://doi.org/10.1080/27697061.2025.2496945>.
7. Pastorino G, Cornara L, Soares S, Rodrigues F, Oliveira MBPP. Liquorice (*Glycyrrhiza glabra*): A phytochemical and pharmacological review. *Phytother Res* 2018;32:2323–39. <https://doi.org/10.1002/PTR.6178>.
8. Hrebien-Filisińska AM, Aydin M, Proch M. The green method of enriching linseed oil with antioxidants as well as flavour and aroma components from herbs and spices. *Int J Food Sci Technol* 2025;60. <https://doi.org/10.1093/IJFOOD/VVAE048>.
9. Cao S, Liang J, Chen M, Xu C, Wang X, Qiu L, Zhao X, Hu W. Comparative analysis of extraction technologies for plant extracts and absolutes. *Front Chem* 2025;13:1536590. <https://doi.org/10.3389/FCHEM.2025.1536590/BIBTEX>.
10. Singh S, Lohani A, Mishra AK, Verma A. Formulation and evaluation of carrot seed oil-based cosmetic emulsions. *J Cosmet Laser Ther* 2019;21:99–107. <https://doi.org/10.1080/14764172.2018.1469769>.
11. Quirin K-W. Phytosterol-Rich Soy Germ and Guggul Extracts Provide Anti-Ageing Benefits. *Natural Ingredients Cosmetic Science Technology* 2011:18.
12. Khalid MU, Sultan MT, Baig I, Abbas A, Noman AM, Zinedine A, Bartkiene E, Rocha JM. A comprehensive review on the bioactivity and pharmacological attributes of *Withania somnifera*. *Nat Prod Res* 2025. <https://doi.org/10.1080/14786419.2025.2499070>.
13. Swayamprabha Chaudhari GZTJYD. Formulation and Characterization of Herbal Cream Prepared from the Extract of Medicinal Plants. *International Journal of Pharmaceutical Sciences* 2025;03:3950–7. <https://doi.org/10.5281/ZENODO.17711240>.
14. Mishra AP, Saklani S, Milella L, Tiwari P. Formulation and evaluation of herbal antioxidant face cream of *Nardostachys jatamansi* collected from Indian Himalayan region. *Asian Pac J Trop Biomed* 2014;4:S679–82. <https://doi.org/10.12980/APJTB.4.2014APJTB-2014-0223>.
15. Rungruang R, Ratanathavorn W, Boohud N, Phakeenuya V, Peasura N, Panichakul T. Anti-elastase, anti-collagenase, and anti-hyaluronidase activities of *Aloe barbadensis* gel extract: In vitro and molecular docking studies. *J Appl Pharm Sci* 2025;15:123–34. <https://doi.org/10.7324/JAPS.2025.235918>.
16. Wang Y, Yu Y, Zhang Y. Therapeutic properties and enzyme inhibition potentials of some natural compounds on hyaluronidase, collagenase, and elastase with molecular docking studies. *Macromolecular Research* 2024 32:5 2024;32:475–91. <https://doi.org/10.1007/S13233-023-00242-6>.
17. Formulation And Evaluation Of Herbal Multipurpose Cream » RTC n.d. <https://journals.stmjournals.com/rtc/article=2024/view=165981/> (accessed January 5, 2026).
18. Sarup P, Bala S, Kamboj S. Pharmacology and Phytochemistry of Oleo-Gum Resin of *Commiphora wightii* (Guggulu). *Scientifica (Cairo)* 2015;2015:1–14. <https://doi.org/10.1155/2015/138039>.
19. Gaurav H, Yadav D, Maurya A, Yadav H, Yadav R, Shukla AC, Sharma M, Gupta VK, Palazon J. Biodiversity, Biochemical Profiling, and Pharmacocommercial Applications of *Withania somnifera*: A Review. *Molecules* 2023;28. <https://doi.org/10.3390/MOLECULES28031208>.
20. Paudel PN, Acharya A, Paudel A, Shrestha A, Satyal P, Setzer WN, Gyawali R. Formulation and evaluation of herbal antioxidant cream from selected plants collected from the Nepalese Himalayan region. *Food Res* 2025;9:8–20. [https://doi.org/10.26656/FR.2017.9\(3\).054](https://doi.org/10.26656/FR.2017.9(3).054).
21. Abdalla S, Aroua MK, Gew LT. A Comprehensive Review of Plant-Based Cosmetic Oils (Virgin Coconut Oil, Olive Oil, Argan Oil, and Jojoba Oil): Chemical and Biological Properties and Their Cosmeceutical Applications. *ACS Omega* 2024;9. <https://doi.org/10.1021/ACSOMEGA.4C04277>.
22. Cervantes Recalde MF, Schmidt J, Girardi C, Massironi M, Rech ML, Hans J, Stuhlmann D, Somoza V, Lieder B. Capsaicin attenuates the effect of inflammatory cytokines in a HaCaT cell model for basal keratinocytes. *Front Pharmacol* 2024;15. <https://doi.org/10.3389/FPHAR.2024.1474898/PDF>.