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Design, development and evaluation of Poly Herbal Anti-aging cream containing *Punica grantum* and *Annona squamosa* leaves extract

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ABSTRACT:

Background: *Punica grantum* and *Annona squamosa* leaves extract has the potential to use in various skin cosmetics as anti-aging creams having antioxidant properties. **Aim:** The present work focuses on exploring the use of natural herbs such as *P. grantum* and *A. squamosa* leaves extract for cosmetic purposes. In this study, the multipurpose-based cream was constructed for cosmetic utility. **Methods:** Various formulation factors were evaluated to find out for stable formulation. The formulations consist of stearic acid 10 %, cetyl alcohol 0.50 %, lanolin, KOH, triethanolamine, and two extracts in different concentrations i.e., 2, 4, and 6 % respectively. **Results:** Topical creams of natural herbs i.e., *P. grantum* and *A. squamosa* were formulated and subjected to physic-chemical studies i.e., pH and spreading coefficient studies. Safety assessment was carried out by a skin irritation test. Formulated multipurpose herbal creams showed acceptable physical properties and antioxidant activity, which remained unchanged upon storage for 3 months. The physical appearance was light yellowish creamy and dark yellowish, pH was between 6 to 6.5, and formulations showed better spreading coefficient. **Conclusion:** It could be concluded that the combination of extract in its low concentration-based creams with the formulation code F7 proved to be the formula of choice since it showed better antioxidant activity i.e., 60.14 %.

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INTRODUCTION:

Cosmeceuticals are cosmetic products with biologically active ingredients purporting to have medical or drug-like benefits. A Cosmeceutical is an ingredient with medicinal properties that manifests beneficial topical actions and provides protection against degenerative skin conditions. The word Cosmeceuticals was popularized by Albert M. Kligman in the late 1970s [1]. It encompasses Cosmetic actives with therapeutic, disease-fighting, or healing properties, serving as a bridge between personal care products and pharmaceuticals. Cosmeceuticals improve appearance by delivering

nutrients necessary for healthy skin. Cosmeceuticals typically claim to improve skin tone, texture, and radiance, while reducing wrinkling [1].

The purported drugs' effects are unproven, and the term is neither recognized by the United States Food and Drug Administration (FDA) nor by any other regulatory body. Nowadays advertisements of many anti-wrinkle and fairness creams are aimed at men. Key cosmeceuticals used by men include hair growth products, anti-aging, antiperspirants, athlete's foot, and astringents. Cosmeceutical active ingredients are constantly being developed for big and wrinkles, anti-cellulite, hair removal, tanning skin whitening, antioxidants, and cell recovery products. Desirable features of cosmeceuticals agents are efficacy, safety, formulation stability, novelty, patent protection, metabolism within the skin, and inexpensive. Small corporations engaged in pharmaceuticals, biotechnology, natural products, and cosmetics, while advances in the field and knowledge of skin cream containing a hormone such as estrogen results in a fresh appearance with a rejuvenating effect [2,4].

Aging is a natural process and the skin represents an ideal marker of chronological age. The exposed skin is subject to environmental damage, particularly that caused by ultraviolet radiation (UVR). Extensive research activities are focused on this skin concern that involves the appearance of unpleasant, observable marks on the skin surface due to proteolysis of cutaneous elastic fibers resulting in reduced cell functions [5,6].

The objective of the proposed study is to evaluate the Poly Herbal Anti-aging cream containing *P. grantum* and *A. squamosa* leaves extract.

MATERIALS AND METHODS:

P. granatum and *A. squamosa* were obtained from the area of Gangakhed City. Stearic acid was procured from S.D. Fine Chemicals, Mumbai. Cetyl alcohol was procured from Alpha Chemicals, Mumbai. All other chemicals are analytical grade and procured from the authorized dealer.

Extraction of *P. granatum* leaves:

Air-dried leaves were ground to a coarse powder in a suitable grinder mixer. The shade-dried powder was extracted using a Soxhlet extractor with hexane and ethanol separately to get a semisolid extract. The organic solvents were recovered by steam distillation. Then extracts were concentrated to dryness under reduced pressure and controlled temperature, respectively and they were preserved in a refrigerator [7].

Extraction of *A. sqamosa* leaves:

Leaves were shade dried at room temperature for 4 to 5 days. The dried leaves were crushed to coarse powder extract was prepared by decoction by boiling leaves in water for 2 h and then filtered by muslin cloth. Then, the liquid obtained was poured into a China dish and heated gently over the water bath. When the liquid gets evaporated to cool the content and scratches it, the dark brown extract was obtained. The extract was stored in a refrigerator.

Determination of Total Flavonoids content of *P. grantum* and *A. squamosa* leaf extract:

The content of flavonoids in the examined plant extracts was determined using the spectrophotometric method [8,9]. The sample contained a solution of the extract in the concentration of 1 mg/ml and 1 ml of 2 % $AlCl_3$ solution was dissolved in methanol. The samples were incubated for an hour at room temperature. The absorbance was determined using a spectrophotometer at λ_{max} is 415 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was noted. The same procedure was repeated for the standard solution of rutin and a dilution series of rutin of concentration 0.01, 0.02, 0.03, 0.04, and 0.05 mg/ml. was prepared and the calibration line was construed. Based on the measured absorbance, the concentration of flavonoids was read (mg/ml) on the calibration line; then, the content of flavonoids in the extracts was expressed in terms of rutin equivalent (mg of RU/g of extract).

Determination of Total Phenolic content:

Total phenolic content was determined using the Folin-Ciocalteu reagent [10]. About 0.1 ml of extract was diluted with 1 ml of distilled water and added to a solution of 0.5 ml of Folin-Ciocalteu reagent and 1.5 ml of 20 % sodium carbonate solution. The reaction mixture was incubated for 2 h, and, finally, the volume was raised to 10 ml, and the absorbance was read at 765nm. Gallic acid (0 to 200 μg/ml) was used for calibration of the standard curve. The total phenolic content was expressed as milligram Gallic acid equivalent (mg GAE/g) dry weight of plant material.

Determination of Microbial count [10].

Microbial evaluation is essential to check the limits of microbial contamination and the extent of pathogenicity.

This evaluation has a direct correlation with the quality of products. The total microbial count was determined by the plate count method.

This test employs a serial dilutions technique for easy quantification of microorganisms.

Preparation of cream:

Oil in water (O/W) emulsion-based cream (Semisolid formulation) was formulated. The emulsifier (stearic acid) and other oil-soluble components (Cetyl alcohol, propylene glycol, and lanoline) were dissolved in the oil phase and heated to 75±5 ºC. The preservatives and other water-soluble components (Methyl paraben, Propyl paraben, Triethanolamine, and potassium hydroxide) were dissolved in the aqueous phase and heated to 75 ºC and then extract added into the aqueous phase once it gets cooled up to 40 ºC. After this, the aqueous phase was added in portions to the oil phase with continuous stirring until the cooling of the emulsifier took place. The perfume was added when the temperature dropped to 45 ºC (Table 1) [3].

Evaluation of cream:

The above-formulated cream formulation was subjected to evaluation of the following parameters.

Physical observation [11]:

Physical parameters such as color, appearance, and feeling on the application were recorded. Color, appearance, and feeling on application and other visual factors like clarity and color of the formulation are the first thing that decides the quality of the formulation.

Homogeneity [12]:

The homogeneity of all developed creams was checked visually for the presence of any aggregates and for appearance.

Spreadability [13]:

Two sets of glass slides of standard dimensions were taken. The herbal cream formulation was placed over one of the slides. The other slide was placed on top of the cream, such that the cream was sandwiched between the two sides in an area occupied by a distance of 7.5 cm along the slide. About 100g weight was placed upon the upper slides so that the cream between the two slides was pressed uniformly to form a thin layer. The weight was removed and the excess of cream adhering to the slides was scrapped off. The two slides in position were fixed to a stand without the slightest disturbance and in such a way that only the upper slide slipped off freely by the force of weight tied to it. A 20 g weight was tied to the upper slide carefully. The time taken for the upper slide to travel the distance of 7.5 cm and separated away from the lower slide under the influence of the weight was noted. Spreadability was calculated by using the following formula.

$$S = M \times L/T \ldots\ldots\ldots\ldots (1)$$

Where, S = Spreadability, L = Length of the glass plate (7.5 cm), M = Weight tied to upper plate (20 g), and T = Time taken to separate the slide completely from each other.

Determination of pH:

The pH of all formulations was determined by a pH meter (Digital pH meter) [14,15]. The pH meter was calibrated with a standard buffer solution having pH 4 and 7 before use. About 0.5 g of the cream was weighed and dissolved in 50.0 ml of distilled water and its pH was measured.

Determination of the type of emulsion [16,17]:

***Dilution test*:**

The small quantity of cream was taken in two test tubes and one was diluted with oil and the other with water separately. Both test tubes were observed for distribution of oil as well as water and stability of an emulsion and result were recorded accordingly.

***Dye solubility test*:**

Scarlet red dye is mixed with cream. Place a drop of cream on a microscopic slide covers it with a coverslip and examines it under a microscope. If disperse globules appear red and the ground is colorless, then it is an O/W emulsion. If it is in reverse, i.e., globules appearing colorless and the ground is red color then it is W/O type. But the result indicated it is an O/W emulsion.

Viscosity:

The viscosity of the cream was determined by the LVT Brookfield viscometer [18]. The sample was placed in a clean and dried container and viscosity was checked as per the standard operating procedure of a viscometer by using spindle no. 4 at a speed of 30 rpm. After recording the dial reading viscosity was calculated in the centipoises (cps). The following formula is used for the calculation of the viscosity.

Viscosity (cp) = Dial reading × Factor….. (2)

For the calculation of viscosity put the factor value corresponding to the speed and the spindle number.

Determination of total fatty matter:

About 2.0 g of the material was accurately weighed into a conical flask, to this 25 ml of dilute hydrochloric acid was added. The contents were refluxed until a solution was perfectly clear. The contents of the flask were poured into a 300 ml separating flask and allowed to cool to 20 ºC. The conical flask was rinsed with 50 ml of ethyl ether in portions of 10 ml. Ether rinsing was poured into the separating flask and the separating flask was shaken. All the ether extracts were combined and washed with water. The ether extracts were filtered through a filter paper containing sodium sulfate into a conical flask which is previously dried at a temperature of 60±2 ºC and then weighed. The sodium sulfate on the filter was washed with ether and washing was combined with filtrate. The ether was distilled off and the material was dried at a temperature of 60±2 ºC to constant mass and total fatty matter was calculated [18].

Test for Thermal Stability [19]:

About 20 mm broad and 5 mm thick strip was spread from the material to be tested on the internal wall of a beaker of 100 ml capacity along its total height. Beaker was kept for 8 h in the humidity chamber at 60 to 70 % relative humidity and temperature of 37± 1 ºC to observe any oil separation on removal from the thermostat.

Determination of the antioxidant activity of formulations:

The test was conducted according to the method of Kikuzaki and Nakatani (1993) [20,21]. To 2.0 ml of the sample solution, 1.0 ml of 20 % aqueous trichloroacetic acid (TCA), and 2.0 ml of 0.67 % aqueous thiobarbituric acid (TBA) solution were added. The final sample concentration was 0.02 % w/v. The mixture was placed in a boiling water bath for 10 min. After cooling, it was then centrifuged at 3000 rpm for 20 min. The absorbance of the supernatant was measured at 532 nm. Antioxidant activity was recorded based on the absorbance of the final day of the assay. Vitamin C is used as a standard in this study.

Skin irritation study [22]:

A set of five mice was used in the study. The cream formulation was applied on the properly shaven skin of mice. Undesirable skin changes, i.e., changes in color and changes in skin morphology were observed for a period of 24 h.

Stability study [22,23]:

The present stability studies are carried out according to guidelines given by The International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH). The cream was filled in a bottle and kept in a humidity chamber maintained at 30±2°C/65±5 % RH and 40±2 °C/75±5 % RH for three months. At the end of the studies, samples were analyzed for their physical properties and viscosity.

Stability Study of Antioxidant Activity of Cosmetic Formulations:

The stability studies on the formulations were conducted whether the antioxidant activity of the formulations would last during the storage period till the formulation is used by consumer. The parameter used for assessing the stability actives was therefore principally the antioxidant activity (Table 2).

RESULTS AND DISCUSSION:

The extract was found to possess physical and chemical properties which were suitable for being used in cosmetic formulations. Where studied for total flavonoid, phenolic content and antioxidant activity of *P. granatum* and *A. squamosa l*eaves extract exhibit fairly good content of flavonoids and phenolic content.

Determination of flavonoid content of Extract:

The flavonoid content of the extract was determined by using Rutin as a standard flavonoid. The Calibration curve was plotted by preparing a solution of rutin (20 to 100 µg/ml). Concentrations are expressed as (Milligram of rutin equivalent/gram of extract). The data is given in Fig 1. The absorbance of *P. grantum* leaves extract is 0.051. The total flavonoid content of the *P. grantum* was found to be 45.3 mg/g of extract. The absorbance of *A. squamosa* leaves is 0.1243. The total flavonoid content of *A. squamosa* was found to be 118.3 mg/g of extract.

Determination of phenolic content:
***Folin Ciocalteu method*:**

The Absorbance of *P. grantum* leaves was 0.5852. The phenol content of *P. grantum* by the Folin-Ciocalteu method was found to be 19.37 mg/g of extract. The data is presented in Fig 2.

Table1. The formulation design of the cream.

Ingredient	F1	F2	F3	F4	F5	F6	F7	F8	F9
Stearicacid	10	10	10	10	10	10	10	10	10
Cetylalcohol	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Lanolin	2	2	2	2	2	2	2	2	2
PotassiumHydroxide	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Propyleneglycol	8	8	8	8	8	8	8	8	8
Triethanolamine	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Propylparaben	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Methyl paraben	0.5	0.50	0.50	0.5	0.5	0.50	0.50	0.5	0.5
PGE	2	4	6	-	-	-	1	2	3
ASE	-	-	-	2	4	6	1	2	3
Water	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s
Perfume	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s

PGE - *P. grantum* extract, ASE - *A. squamosa* extract, and q.s. – Quantity sufficient.

Table 2. The percentage inhibition of Vitamin C, Flavonoid content, and CT.

Conc. (µg/ml)	20	40	60	80	100	ICV_{50}
Ascorbic Acid	35.23±0.3	51.80±0.5	61.71±0.3	64.95±0.3	77.14±0.7	43.60±0.71
PG Ethanolic extract	24.57±0.9	31.23±0.4	40±0.13	56.57±0.8	60±0.77	75.46±1.07
AS Aqueous Extract	7.42±0.89	24.57±0.7	35.23±0.9	44.95±0.8	54.28±0.3	85.39±0.65

PG - *P. grantum* and AS - *A. squamosa*. ** = Statically Significant of p<0.01, with compared to Ascxorbic acid. Each data is represented as Mean ± Standard deviation, n=3.

Table 3. Determination of physical properties of formulated Creams.

Formulation	Colour	Homogeneity	Consistency	Phase separation
F1	Light yellowish creamy	Homogeneous	Cream like semisolid	No
F2	Light yellowish creamy	Homogeneous	Cream like semisolid	No
F3	Light yellowish creamy	Homogeneous	Cream like semisolid	No
F4	Light yellowish creamy	Homogeneous	Cream like semisolid	No
F5	Light yellowish creamy	Homogeneous	Cream like semisolid	No
F6	Light yellowish creamy	Homogeneous	Cream like semisolid	No
F7	Light yellowish creamy	Homogeneous	Cream like semisolid	No
F8	Light yellowish creamy	Homogeneous	Cream like semisolid	No
F9	Light yellowish creamy	Homogeneous	Cream like semisolid	No

Evaluation of Cream:

***Physical appearance of cream formulation*:**

The prepared cream formulations were inspected visually for their color, homogeneity, and consistency. The data is given Table 3.

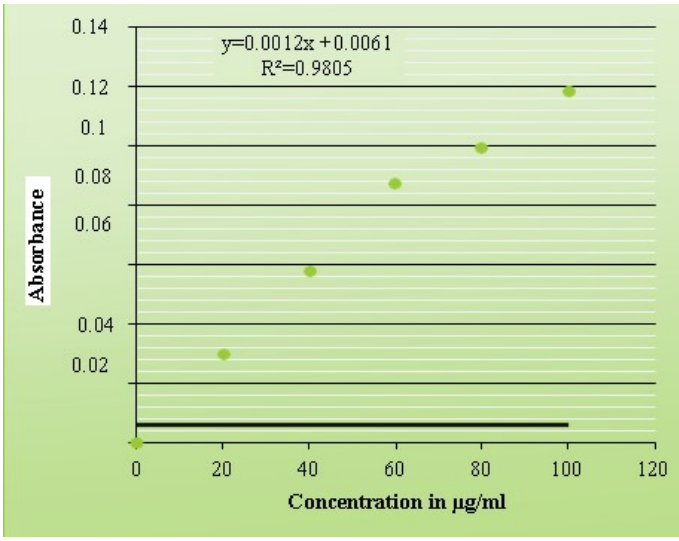

Fig 1. The standard curve of Rutin.

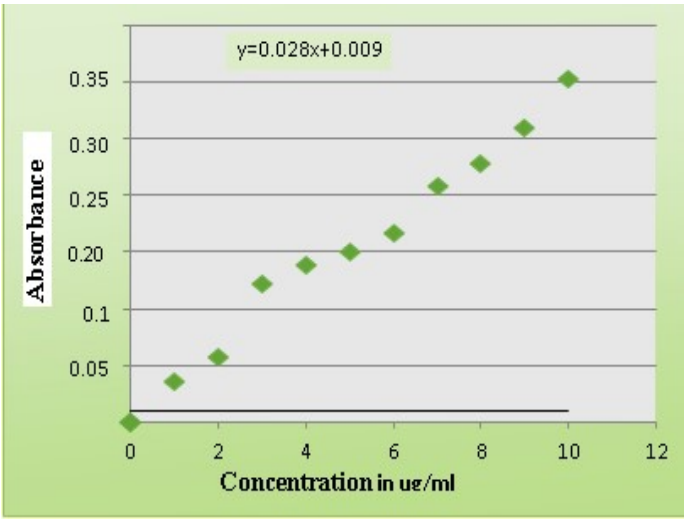

Fig 2. The standard curve of Gallic acid.

***Viscosity*:**

The viscosity of all batches was found to be within the range of 2.17 to 2.76 × 10^4 cps which indicates that cream is easily Spreadable by applying a little shear. Also, facility ease of application. From all of above the batches F7 gives acceptable viscosity, as per the data given in Table 4.

***Antioxidant activity of Cosmetic formulations*:**

The antioxidant activity of all batches was carried out with the Thiobarbituric acid method. The inhibition values of all batches were found within the range of 32.95 to 69.90 %. This was near Vit C. However, batch F7 proved to be the formula of choice, since it showed better antioxidant activity i.e., 60.14 %, as given in the data in Table 5.

***Determination of pH*:**

The pH of the topical formulations should be compatible with skin pH. A change in the pH may cause skin irritation or disruption. The pH of the all-cream formulations was modified with the help of triethanolamine and when checked, it was found in between the range of 6 and 6.5, which is acceptable for skin preparations (Table 6).

***Spreadability*:**

Spreadability is the term expressed to denote the extent of the area to which the cream readily spreads on application to the skin. One of the essential criteria for a cream is that it should have good spreadability. It depends upon the type and concentrations of emulsifier used in the formulation. A more viscous formulation would have poor spreadability, as shown in Fig 3. The spreadability of batch F1 to F9 was in the range of 11.24 to 12.3 (Table 6).

***Skin irritation test*:**

The skin irritation study was carried out to check the possible sensitivity of cream using mice. The results of the skin irritation test for creams are depicted in Table 7. The tests showed no irritation and sensitization potential of all formulations on animals.

***Stability study*:**

The stability test for the optimized formulation (F7) was carried out for three months and results revealed that all the creams show better stability and 40 and 30 °C. There were no significant changes found in color, homogeneity, consistency, and phase separation at all times. Also, the pH, spreadability thermal stability, and viscosity of optimized batch F7 were not shown any makeable changes at all times as shown in Table 8.

***Comparison with the marketed product*:**

The comparison between the optimized anti-aging cream and the marketed cream (Patanjali Saundarya Anti-aging Cream) is depicted in Table 9. All the results of optimized batch F7 were found to be similar to the marketed Patanjali Saundarya anti-aging cream. So, it can be concluded that crams containing extracts have antioxidant properties and can be used as the provision barrier to protect the skin.

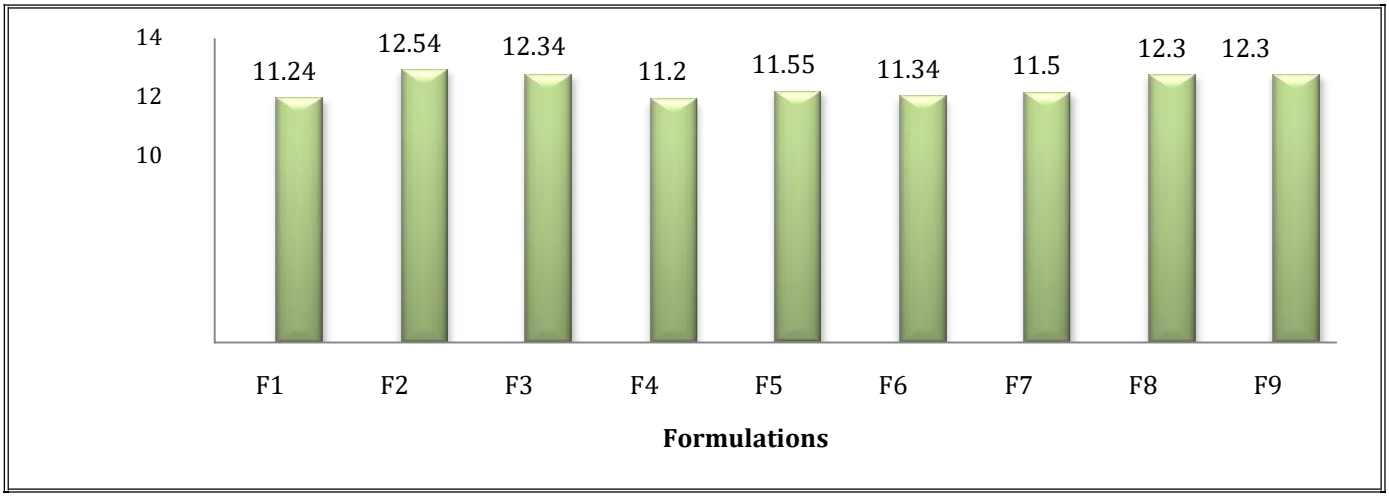

Fig 3. The spreadability of formulated creams.

Table 4. Determination of viscosity of formulated creams.

Formulation	RPM				Viscosity (cps)
	6	12	30	60	
F1	42	45	62	100	21725±19.20
F2	48	55	77	100	25225±31.62
F3	45	52	82	100	24350±26.24
F4	40	42	50	95	20125±35.66
F5	43	50	62	100	22600±20.41
F6	48	57	64	100	24887±12.55
F7	47	52	60	97.5	23750±18.50
F8	50	66	87	100	27600±21.60
F9	48.5	59	88.5	100	26425±18.70

Viscosity value is represented as Mean ± Standard deviation (n=3).

Table 5. Inhibition of Thibarbituric acid activity.

Batch	Control	Absorbance	Inhibition (%)
F1	0.0525±0.0004	0.0352±0.0008	32.95
F2	0.0525±0.0004	0.0258±0.0002	50.85
F3	0.0525±0.0004	0.0217±0.0004	58.66
F4	0.0525±0.0004	0.0321±0.0007	38.85
F5	0.0525±0.0004	0.0209±0.0005	60.19
F6	0.0525±0.0004	0.0206±0.0002	60.76
F7	0.0525±0.0004	0.0204±0.0004	60.14
F8	0.0525±0.0004	0.0199±0.0001	62.09
F9	0.0525±0.0004	0.0158±0.0004	69.90
Vitamin C	0.0525±0.0004	0.0120±0.0002	77.14

The values are represented as Mean ± Standard deviation (n=3).

Table 6. Determination of physical properties of formulated creams.

Formulation	pH	Spreadability (g.cm/s)	TFC (%)	Type of emulsion
F1	6.40	11.24±0.06	8	O/W
F2	6.45	12.54±0.02	10	O/W
F3	7.00	12.34±0.04	14	O/W
F4	6.36	11.20±0.04	9.2	O/W

F5	6.40	11.55±0.06	12	O/W
F6	6.38	11.34±0.03	12	O/W
F7	6.20	11.50±0.04	9	O/W
F8	6.40	12.30±0.08	10	O/W
F9	6.48	12.30±0.02	10	O/W

TFC – Total Flavonoid content. The spreadability values are represented as Mean ± Standard deviation (n=3).

Table 7. The animal skin irritation test.

Group	Time (h)	Erythma	Edema
1 (Control)	24	0	0
2 (Cream with drug)	24	0	0
3 (Cream without drug)	24	0	0

Table 8. The stability study results of F7 formulation.

Temperature (°C)	Day	pH	Viscosity	Spreadability	Thermal stability
40	1	6.20	23750±18.50	11.50±0.04	NOS
	15	6.19	23748±17.80	11.48±0.10	NOS
	30	6.19	23747±18.20	11.45±0.08	NOS
	60	6.15	23745±18.39	11.43±0.04	NOS
	90	6.13	23738±18.16	11.40±0.09	NOS
30	1	6.20	23750±18.50	11.50±0.04	NOS
	15	6.18	23748±18.40	11.46±0.09	NOS
	30	6.16	23747±18.20	11.45±0.07	NOS
	60	6.13	23745±19.27	11.43±0.05	NOS
	90	6.12	23742±18.18	11.41±0.01	NOS

NOS – No oil separation. The values are represented as Mean ± Standard deviation (n=3).

Table 9. Comparison of marketed cream with F7 formulation.

Evaluation Parameter	Optimized Cream (Batch F7)	Marketed cream
pH	6.20±0.02	6.21±0.02
Spreadability	11.50±0.04	12.42±0.02
Viscosity (cps)	23750±18.50	25652±16.25
Anti-oxidant activity (% Inhibition)	60.14 %	64.25 %

The values are represented as Mean ± Standard deviation (n=3). Marketed cream - Patanjali Saundarya Anti-aging Cream.

CONCLUSION:

From all these findings, the present work concluded that formulated cream of these two extracts has the potential to use for functional cosmetics. *Punica grantum* and *Annona squamosa* leaves extract has the potential to use in various skin cosmetics. However, to enhance the skin cosmetics utility of these two extracts there is a need for further study in the future to isolate active compounds from them for various functional applications.

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