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Effect of Pumpkin Seed Oil for the Treatment of Androgenetic Alopecia: Formulation and In-Vitro Evaluation

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Abstract: ***Objectives: To formulate and evaluate a pumpkin seed oil hair gel for the treatment of androgenetic alopecia. Methods: Pumpkin seed oil has a broad spectrum of anti-androgenic properties, making it appropriate for the treatment of androgenetic alopecia. Pumpkin seed oil gel is composed of Carbopol 940 and HPMC K100 as gelling agents, pumpkin seed oil as an active ingredient, propylene glycol as a co-solvent, and ethanol as preservative and required quantity of distilled water as vehicle. Results: Physical parameters like FT-IR study of pumpkin seed oil showed satisfactory results. The prepared gel was tested for various properties like pH, drug content, clarity, viscosity, extrudability, spreadability etc. In-vitro release and cell cytotoxicity studies proved that formulation F3 is an optimized one that is non-irritant. Conclusion: Based on the present study, gel formulations of pumpkin seed oil F3 had excellent physicochemical properties and in vitro drug release in comparison to other formulations.***

Keywords: Pumpkin seed oil, Carbopol 940, HPMC K100, androgenetic alopecia

1. Introduction

Luscious hair is associated with youth, attractiveness, and good health in almost every culture on the world. Alopecia or hair loss, affects both men and women equally. Finasteride, minoxidil, hair replacements, and hair restoration are all options for treating hair loss.[1]

Most common type of alopecia, which affects both men and women, is androgenetic alopecia (AGA).it is a disorder attributed to testosterone. Hair follicles are continuously miniaturized during the process. The enzyme 5 alpha-reductase is accountable for the intrafollicular conversion of free testosterone to dihydrotestosterone (DHT), which is a key element to hair loss, characterized by smaller anagen phases and shortening of hair strands.[2,3]

Pumpkin seeds are commonly dumped as agro-industrial waste. In many part of the world, the seeds are eaten raw, roasted, or cooked in minimal amount. Pumpkin seed oil provides a good source of unsaturated fatty acids, phytoestrogens, and vitamins E, with potential medicinal, nutraceutical, and cosmeceutical applications. The amount of knowledge obtainable on the nutritional components and therapeutic effects of pumpkin seeds oil has rapidly increased in recent years. They are currently considered beneficial for culinary goods due to their high protein, minerals, fiber, polyunsaturated fatty acids, and phytosterol content. They are also being studied for their potential benefits to blood glucose levels, resistance, lipids, the liver, the endocrine gland, the kidney, anxiety, developmental delays, and parasite suppression. Converting these agricultural wastes into significance components is expected to be a significant move forward in global initiative towards sustainability, and it merits further investigation.

Alopecia can be effectively treated with pumpkin seed oil (PSO), obtained from pumpkin seeds. Phytosterols account for its effects, which have been demonstrated in rats to inhibit 5α-reductase and be anti-androgenic. The 5 alpha reductase enzyme takes some testosterone and converts it into Dihydrotestosterone (DHT). DHT is related to prostate growth, acne, facial hair growth, and baldness. It is suppressed by 5α reductase inhibitors.[4, 5]

Researchers reported that taking Pumpkin seed oil 400 mg (PSO) orally for 24 weeks enhanced hair growth and had antioxidant, antimicrobial, anti-inflammatory, cytoprotective and anti-diabetic effects on men with androgenic baldness. Topical application of PSO would be preferred over oral administration due to its side effects such as bloating and constipation.[6]

The aim of this work is to develop hair gel with PSO (extracted from pumpkin seeds of *Curcubita pepo*) using soxhlet extraction method and utilized in the formulation along with Hydroxypropyl methylcellulose (HPMC K100) and Carbopol 940 as polymer. Various gel formulations are prepared with polymer (HPMC K100 and Carbopol 940) and in-vitro evaluations, like drug release, Gel strength, spreadability, in vitro skin irritant test and stability studies are assessed. Subsequently, the best formulation is selected based on the study report. Estimated outcome of this hair gel is to provide rapid treatment of Alopecia and prevent hair loss by the inhibitory effect against 5α reductase enzyme.

2. Literature Survey

1) **Ibrahim et al., 2021** reported that clinical efficacy of pumpkin seed oil (PSO) in the treatment of female pattern hair loss (FPHL) and compare its effects with minoxidil 5 % foam. Conclusion findings of the trial provide evidence of a promising potential role of PSO in treating FPHL.[16]
2) **Hajhashemi et al., 2019** reported that oral administration of pumpkin seed oil improved hair

growth in male pattern alopecia. Concluded that topical application of PSO showed hair growth promotion, it might be regarded as a promising alternative for treatment of male pattern alopecia.[17]

3) **Young Hye Cho et al., 2014** reported that pumpkin seed oil (PSO) has been shown to block the action of 5-alpha reductase and to have anti-androgenic effects on men.[6]
4) **Parhi R, et al., 2014** developed topical gel of minoxidil using model polymers such as hydroxypropyl methylcellulose K4M (HPMC K4M) and hydroxypropyl cellulose (HPC) at different concentrations (1, 2 and 3 %) individually and in combination.[7]
5) **Kim MY, et al., 2012** reported that *Cucurbita pepo* seeds had significantly more β-sitosterol than *Cucurbita maxima* and *Cucurbita moschata*.[18]
6) **D. Jı´rova´ et al., 2003** reported that cytotoxicity tests represent a highly ethical approach for estimation of irritancy. On the basis of in vitro test results suggesting low risk we can proceed to confirmatory tests in human volunteers.[19]

3. Methodology

Material:
Pumpkin seed oil was extracted and collected from Helikem biotek industrial research Pvt. Ltd, Trichy; Carbopol 940 was purchased from Kemphasol, Mumbai; HPMC K100 was purchased from National chemicals, Maharastra; Ethanol was purchased from Nice chemicals Pvt. Ltd, Kerala; Triethanolamine and propylene glycol were purchased from Loba Chemie Pvt. Ltd Mumbai.

FT-IR studies:
The infrared spectrum of any chemical provides structural information. The IR spectral data of the drug and physical additives of drug with different excipients were complied and analysed for drug-specific peaks using the KBr disc method (Schimadzu IR) in the range of 4000-400 cm^{-1}.

Procedure of PSO hair gel
Approximately 4ml of accurately weighed Pumpkin Seed Oil (equivalent to 4gm) is dissolved in a solvent mixture (30% ethanol, 15% propylene glycol, and 43 - 46 % water).For around 2 hours, the specified amount of polymer (Carbopol 940 and HPMC K 100) was added to the solution while it was constantly stirred on a magnetic stirrer at 500 rpm. The speed was later lowered to minimize air entrapment. The pH of the medium was then modified by neutralising it with triethanolamine.[7]

Table 1: Formulation of PSO hair gel

Ingredients	F1	F2	F3	F4	F5	F6	F7
Pumpkin seed oil(PSO) (ml)	4	4	4	4	4	4	4
HPMCK 100(gm)	0.5	1	0.25	-	-	0.25	0.5
Carbopol 940(gm)	-	-	0.25	0.5	1	0.5	0.25
Ethanol(ml)	15	15	15	15	15	15	15
Propylene glycol (ml)	7.5	7.5	7.5	7.5	7.5	7.5	7.5
Triethanolamine(ml)	q.s	q.s	q.s	q.s	q.s	q.s	q.s
Water(ml)	23	22.5	23	23	22.5	22.75	22.75

Characterization of gel
Gels are evaluated using conventional procedures for pH, clarity, homogeneity, extrudability, drug content, spreadability, viscosity, in-vitro diffusion studies, release kinetics, and stability studies. The studies were repeated three times, and the averages were provided.

Clarity:
Clarity of many formulations was assessed visually against a black and white backdrop and classified as turbid, clear, or very clear (glassy).[7]

pH:
In 25 ml of purified water, 2.5 gms of gel are precisely weighed and distributed. A digital pH metre (Elico) is used to determine the pH of the dispersion.[7]

Homogeneity:
Gel homogeneity is determined visually after they have been packed in the container to check whether aggregates are present.[8]

Extrudability:
The Pfizer hardness tester is used to perform the extrudability test. In an aluminium tube, 15gm of gel is placed. The plunger has been adjusted to adequately retain the tube. For 30 seconds, a pressure of 1 kg/cm2 was applied. The amount of gel extruded is weighed. The technique is performed at three equidistant points along the tube. The test was performed in triplicate.[9]

Spreadability:
As a measure of the spreadability, 0.5 g of the formulation was deposited on two smooth surface glass plates and the diameter at which it spreads was measured. Initially, the diameter of the gel on the glass plate was measured in centimeters. Another 200-g glass plate with the same dimensions was placed over the gel for one minute or until there was no further expansion. The upper plate was gradually removed, and the diameter of the circle created after spreading the gel was measured in centimeters.[8, 9]

Viscosity measurement:
Viscosity is determined using a Brookfield viscometer, (Brookfield DV-II + Pro viscometer) by using spindle number SC4- 18/13R, by applying torque ranging from 10 to 100 %.[8,9]

Drug content:
In 50 ml of phosphate buffer 7.4, hair gel in the amount of 100 mg was dissolved. The graduated flask with the solution was mechanically shaken for two hours to ensure that the drug was fully dissolved. It was filtered and measured spectrophotometrically.[9]

In-vitro drug release studies:
Diffusion process determines in-vitro release rate of Pumpkin seed oil from hair gel. 1 gm of hair gel is stored in the donor compartment over an egg membrane that has been cleaned and immersed in the diffusion medium for 24 hours. The donor compartment is submerged in the receptor compartment, which contains 400ml of phosphate buffer with pH 7.4. The diffusion medium (receptor compartment)

is maintained at 37° C and mixed with a magnetic stirring mechanism at 22 rpm. Every hour for the next 8 hours, 5 ml aliquots are removed from the diffusion medium and restored with the same amount of new, pre-warmed diffusion material. Shimazdu Double beam UV- Visible spectrophotometer was used to analyse the samples with drawn at 264 nm for the Pumpkin seed oil. All seven formulations were evaluated with relation to a diffusion analysis, as well as their release characteristics were analysed using a kinetic model.[9, 10, 11]

Drug release kinetics:
Various kinetic models, such as the zero order, first-order, Higuchi, and Korsemeyer-Peppas equations, are applied to in vitro drug release data to better understand the drug release process.[10, 11]

MTT assay for cell cytotoxicity:
MTT assay is used to evaluate the cytotoxicity of the formulation using 3T3 cells. A trypsinization procedure is used to extract cultivated 3T3 cells, which are then collected in 15 ml vials. The cell lines are then mounted into a 96-well cell culture plate at a density of 1×10^5cells/ml cells/well (200 µl) in DMEM medium containing 10% FBS and 1% anti-biotic for 24 - 48 hours at 37° C. The wells in a serum-free DMEM medium are rinsed with sterile Phosphate Buffered Saline (PBS) before being administered with different amount of the sample. Cells are cultured in a humidified 5% CO_2 incubator for 24 hours at 37° C for every sample. Following inoculation, MTT (20 µl at 5 mg/ml) is added to each well, and cells are inoculated for another 2 to 4 hours, or until purple filtrate scan be seen under inverted phase microscope. The medium and MTT (220 µl) are then evacuated from the wells and rinsed with 1X PBS (200 µl). In addition, DMSO (100 µl) is added to disperse formazan crystals and the plate is agitated over five minutes. Microplate reader (Thermo Fisher Scientific, USA) is used to measure absorbance of each well and Graph Pad Prism 6.0 software (USA)is used to compute percentage of cell viability and IC50 value.[12,13,14]

Percentage of Cell viability = Test OD/Control OD X 100

Stability studies:
The optimized Pumpkin seed oil hair gel is packed in a suitable container and kept in a stability chamber at 40˚C/75 % Relative Humidity. Pumpkin seed oil hair gel is evaluated for physical characteristics, In vitro drug release and stability after three months of storage.[15]

4. Result and Discussion

FT-IR studies:
The FT-IR graph of drug, polymer and physical combination of drug and polymer was taken. Physical mixture demonstrated that there was no shifting of functional peaks, and all of the key peaks present in the pure drug spectrum were clearly visible in the physical mixture spectrum. There was no evident interaction between the medication and the excipients.

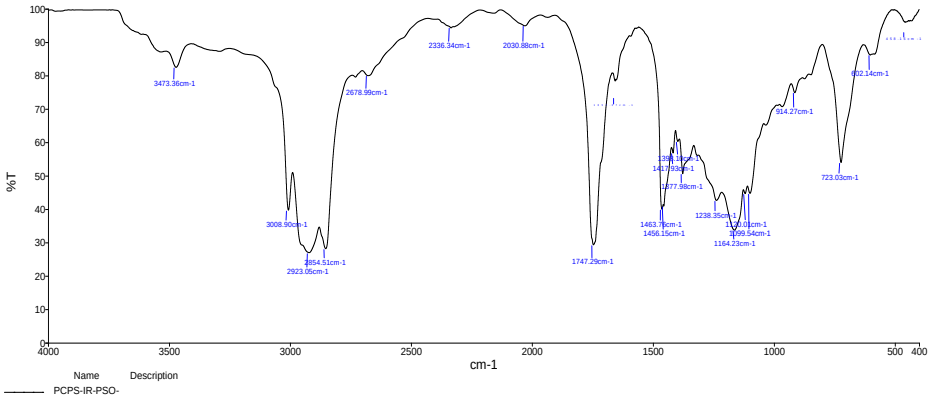

Figure 1: FT-IR spectrum of PSO

Clarity:
The visual appearance of the formulation is an essential feature as it influences patient compliance. All of the formulations were examined visually, and the formulations F1 and F2 were clear, whereas the formulations F3-F7 were opaque.

pH:
The pH of all formulations was found to be adequate, ranging from 6.7 to 7.3. This is regarded to be close to the pH of the skin and is suitable for application on skin with minimum risk of tissue irritation.

Homogeneity:
Hair gels were developed with polymers that were totally soluble in the solvent system; hence, the final formulations were homogeneous and free of lumps.

Extrudability:
Extrudability results were adequate for all F4, F5, F6, sand F7 formulations and outstanding for F1, F2, and F3formulations.

Spreadability:
The diameters of gels spreading as a result of the Spreadability test range between 10 and 22g.cm/sec. This implies that the spreadability increased as the polymer content decreased.

Viscosity measurement:
The rheological properties of the formulation are important in topical dosage form. The viscosity of the formulation increased significantly as the polymer concentration in the formulation increased. The average viscosities of all

formulations were determined to be between 7800 and 10000 Cps.

Drug content:

Drug content of PSO hair gel formulations was observed to be between 74 - 93 %, demonstrating homogeneous oil distribution in the formulation. The maximal drug content of the formulation F3 was determined to be 93.52 %, demonstrating homogenous drug distribution and insignificant drug loss during the formulation. All the parameter values were shown in Table 2.

Table 2: Physiochemical evaluation of formulations

Formulations	Clarity	pH	Homogeneity	Spreadability (g.cm/sec)	Extrudability	Viscosity (cps)	% Drug Content
F1	Clear	6.9	Good	22.08	++	7823	74.92
F2	Clear	7.1	Good	20.56	++	8118	85.20
F3	Turbid	7.3	Good	19.21	++	9951	93.52
F4	Turbid	6.8	Good	17.56	+	9890	78.59
F5	Turbid	6.9	Good	10.85	+	9932	84.82
F6	Turbid	6.7	Good	15.35	+	9826	78.06
F7	Turbid	7.0	Good	12.29	+	9742	75.82

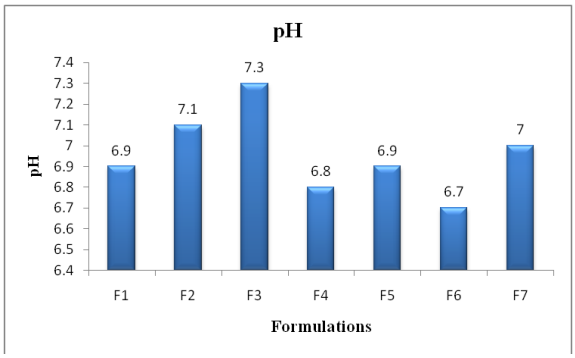

Figure 2: Comparative pH of formulations

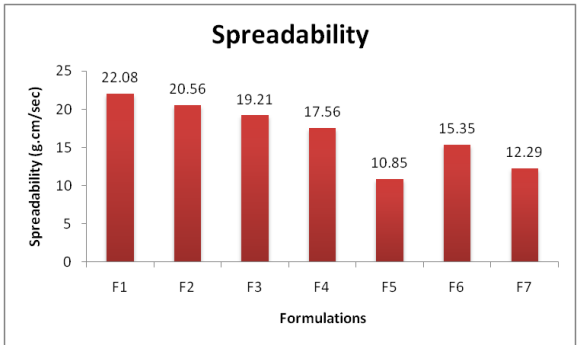

Figure 3: Comparative spreadability of formulations

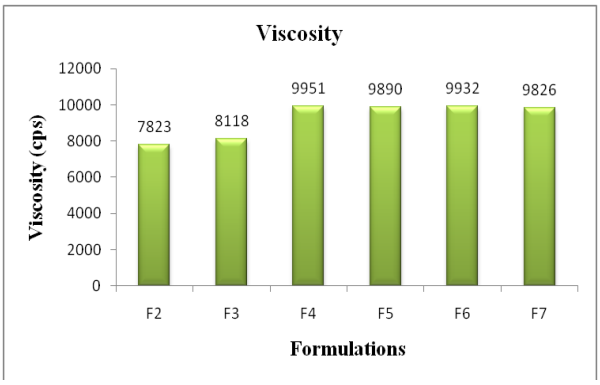

Figure 4: Comparative viscosity of formulations

In-vitro drug release studies:

The results of an 8-hour In-vitro release study of PSO-containing hair gel from all formulations in phosphate buffer pH 7.4 are shown in Table 17. With increasing polymer concentration, the rate and extent of drug release reduced considerably. According to the results of the study, HPMC K 100 impacts the release rate and reduces the release to some extent, while formulations containing Carbopol 940 exhibited better release. According to the diffusion study, formulation F3 has the highest percent drug release (76 ± 0.52) than the other formulations. Based on the results of the study, F3 was chosen as an optimal formulation for further investigation.

Table 3: Comparative in-vitro diffusion studies of formulations

Time (mins)	Cumulative percentage of drug release						
	F1	F2	F3	F4	F5	F6	F7
5	1.32± 0.10	2.61± 0.66	3.21± 0.34	1.41± 0.07	1.23± 0.13	1±0.34	1.63± 0.34
15	5.04± 0.56	8.4±0.11	9.03± 0.14	6.2±0.22	7.81± 0.45	7.21± 0.52	8.87± 0.23
30	9.11± 0.12	12.9± 0.21	14.7± 0.56	10.0± 0.18	10.2± 0.28	9.8±0.36	12.64± 0.48
60	12.5± 0.18	18.6± 0.56	20.3± 0.43	14.6± 0.37	18.7± 0.39	17.9± 0.12	19.24± 0.10
120	20.4± 0.22	26.7± 0.25	29.4± 0.16	22.8± 0.23	24.0± 0.66	23.5± 0.35	25.85± 0.34
180	28.2± 0.35	30.2± 0.18	31.6± 0.32	29.9± 0.16	26.6± 0.32	25.8± 0.23	29.83± 0.56
240	32.4± 0.11	36.4± 0.36	38.5± 0.28	34.8± 0.25	33.2± 0.11	32.6± 0.26	35.72± 0.37
300	37.6± 0.53	42.2± 0.27	44.0± 0.56	38.1± 0.54	38.4± 0.34	37.3± 0.42	40.91± 0.21
360	41.2± 0.21	54.8± 0.31	56.3± 0.18	43.6± 0.32	47.6± 0.56	46.9± 0.14	52.51± 0.38
420	52.8± 0.34	59.6± 0.11	65.4± 0.24	54.2± 0.18	53.3± 0.15	52.9± 0.23	58.74± 0.46
480	58.1± 019	65.8± 0.43	76.0± 0.52	60.8± 0.26	59.3± 0.47	58.5± 0.13	64.22± 0.11

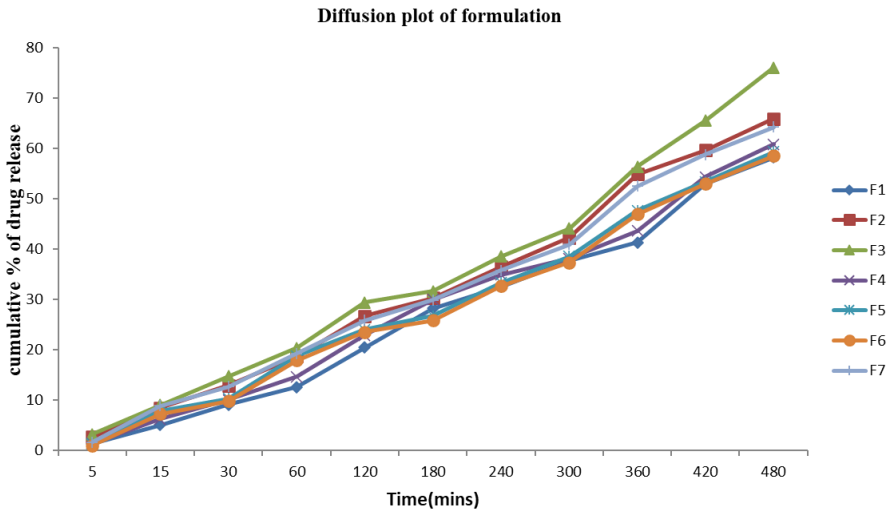

Figure 5: Comparative in-vitro diffusion studies of formulations

Drug release kinetics:

The release kinetics research of the formulation F3 was performed out, as indicated in Table 18 and fig. 27 – 30. F3 has zero order kinetics and a non-fickian diffusion mechanism, according to the release kinetics. Table 18 demonstrates this point.

Table 4: Release kinetic studies of F3 formulations

Formulation code	Zero	Higuchi	Peppas		First
	r^2	r^2	r^2	N	r^2
F3	0.973	0.956	0.9669	0.609	0.9432

MTT assay for cell cytotoxicity:

Cell cytotoxicity assay for the F3 sample was carried out on 3T3 cell lines. The cell viability effect of F3 against 3T3 cell lines at different concentrations was examined using the MTT assay after 24 hours, and the results are described in Table 19, with the control mean OD value derived as 0.481 from Table 5.

Table 5: OD value at 570nm

S. No.	Tested sample concentration (µg/ml)	OD value at 570 nm (in triplicates)		
1	Control	0.623	0.589	0.585
2	500 µg/ml	0.211	0.217	0.211
3	400 µg/ml	0.298	0.297	0.289
4	300 µg/ml	0.351	0.352	0.37
5	200 µg/ml	0.391	0.389	0.387
6	100 µg/ml	0.431	0.428	0.424
7	80µg/ml	0.463	0.461	0.467
8	60µg/ml	0.489	0.481	0.483
9	40 µg/ml	0.534	0.523	0.521
10	20 µg/ml	0.599	0.565	0.564
11	10 µg/ml	0.612	0.579	0.576

In-vitro skin irritation data can be gathered from the findings of the Cell Cytotoxicity (MTT) experiment. According to OECD guidelines, irritating chemicals are defined by their potential to reduce cell viability below threshold levels (50 %), while test compounds that create cell viabilities over the established threshold may be classified non-irritants (> 50%). According to Table 6, F3 concentrations ranging from 10g/ml to 500g/ml have cell viability more than 50 %, showing that F3 does not cause skin irritation.

All concentrations of the sample do not induce cell cytotoxicity as illustrated and the 50-percentage inhibitory concentration (IC50) for the sample also determined as 1043.6 µg/ml.

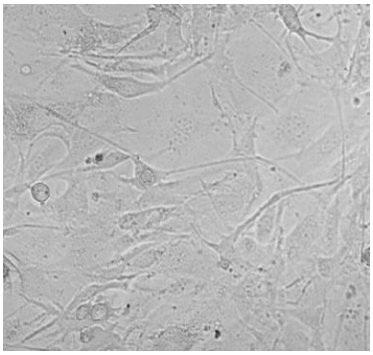

Figure 6: Control cell

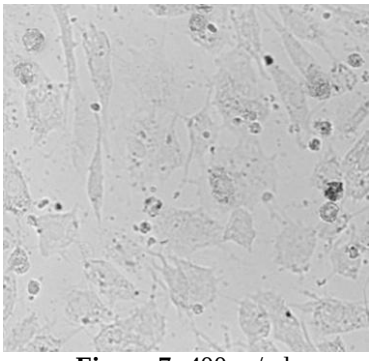

Figure 7: 400µg/ml

Table 6: Cell viability % of F3

S. No	Tested sample concentration (µg/ml)	Cell viability(%)(in triplicates)			Mean Value (%)
1	Control	100	100	100	100
2	500 µg/ml	43.8684	46.8421	46.0684	45.592953
3	400 µg/ml	47.8331	50.4244	49.4017	49.219741
4	300 µg/ml	56.3403	59.7623	63.2479	59.783487
5	200 µg/ml	62.7608	66.0441	66.1538	64.986274
6	100 µg/ml	69.1814	72.6655	72.4786	71.441849
7	80µg/ml	74.3178	78.2683	79.8291	77.471709
8	60µg/ml	78.4912	81.6638	82.5641	80.90637
9	40 µg/ml	85.7143	88.7946	89.0598	87.856227
10	20 µg/ml	96.1477	95.9253	96.4103	96.161075
11	10 µg/ml	98.2343	98.3022	98.4615	98.332699

Stability studies:

The optimized formulation F3 stored in wide mouth plastic container and stored in humidity chamber at 40º ± 2º C and 75 ± 5 % RH for 6months. After 30-, 60- and 90-days samples are analyzed for the determination of pH, viscosity, spreadability, drug content and drug release. It was observed that there were no significant changes before and after the stability study and it was shown in Table 7.

Table 7: Various characteristics of F3formulation before and after stability study

S. No	Parameter	Observation						
		Initial	At the end of 1st Month		At the end of 2nd Month		At the end of 3rd Month	
			RT	40º±2ºC & RH 70±5%	RT	40º±2ºC & RH 70±5%	RT	40º±2ºC &RH 70±5%
1.	pH	7.3	7.1	7.2	7.2	7.2	7.2	7.2
2.	Spreadability	19.21	19.21	19.21	19.20	19.20	19.20	19.20
3.	Extrudability	++	++	++	++	++	++	++
4.	% Drug content	93.5	92.5	93.0	92.5	92.5	92.5	92.5
5.	% Drug release	76.03	75.04	76.02	75.04	76.02	75.0	76

5. Conclusion

The present work has achieved the objective of formulating a safe, cost efficient and effective topical hair gel targeted to treat androgenetic alopecia. It was concluded based on the study; the prepared hair gel produces sustained drug delivery in extended period of time (8 hrs). However, diffusion study should be continued to determine the maximum drug release and Clinical studies are necessary in the same direction to enhance the therapeutic efficacy and improve the patient compliance.

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