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STATISTICAL OPTIMIZATION OF HERBAL CREAM CONTAINING WOUND HEALING AND ANTIFUNGAL AGENTS

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ABSTRACT
Different fungi are always present on surface of the skin. Infection occurs when these organisms enter into body. Fungal infection is a very common ailment of day to day life. Wound refers to a sharp injury which damages the dermis of skin. Microbes i.e. bacteria or fungi are found on wounds. Alkaline wound fluid will promote growth of both bacteria and fungi. The aim of the research is to formulate a cream which treats fungal infection as well as heal wounds which occur due to intense scratching and itching. Conventional therapy shows varied side effects and thus it is need of hour to find an alternative therapy. Herbal therapy is the best option here. Thus, the present study focuses on preparation of methanolic extract and herbal oil and formulating it in a patient friendly formulation i.e. Cream. The cream was formulated and optimized by using 3^2 factorial design with concentration of stearic acid and tween 80: span 20 as factors and viscosity, spreadability and drug release as responses. The cream with 4% tween 80: span 20 and 2 gram stearic acid was considered as optimized. The optimized cream showed maximum drug release through drug (karanj oil) present in oil phase and cream was found to give good results.

KEYWORDS: Karanjin, Rhein, Antifungal and Wound Healing Activity.

INTRODUCTION
Fungi are eukaryotic (contain a true nucleus) organisms that lack chlorophyll and rely on preformed organic matter as their energy source. Fungi are grouped into two broad categories-yeasts and molds-based on their physical characteristics (single cells or hyphae) and mode of reproduction (budding or spore formation). In general, fungi grow in damp, dark environments. The specific type of fungi present in a particular area depends on environmental conditions and available substrates. Of more than 100,000 fungal species, only several hundred cause infection and disease in humans. Fungi are found ubiquitously in the environment; however, despite this constant exposure, serious fungal diseases are rarely found in immune competent individuals. Fungal infections, called mycoses (*myco* Latin for fungus) may be superficial, deep, or systemic, and may cause very mild to life-threatening disease.[1]

The most common types of fungal infections are superficial skin infections caused by a variety of fungal species known as dermatophytes. Chronic fungal infection of the skin, hair, or nails is caused by specific species of fungi such as Trichophyton, Microsporum and Epidermophyton. In layman's terms, the condition is called "ringworm" or tinea infection which is extremely common in general practice. Ringworm is characterized by round lesions (rings) and the word Tinea is used to describe a group of contagious skin infections caused by a few different types of fungi. They can affect many areas of the skin and depending on their location and fungal type, there are multiple terms for tinea infection of various body sites such as; tineacorporis (body), tineapaedis (feet), tineaunguium (nail), tineacapitis(scalp) or tineacruris (groin).[2]

Due to scratching of wounds in fungal infections wounds may develop. Wounds are physical injuries that result in an opening or breaking of the skin. Wound may be defined as a loss or breaking of cellular and anatomic or functional continuity of living tissues. Healing is a complex biological process initiated in response to an injury that restores the function and integrity of damaged tissues. Microbes i.e. bacteria or fungi are found on wounds. Alkaline wound fluid will promote growth of both bacteria and fungi. Microbial infection of wounds delays healing and causes a more pronounced acute inflammatory reaction which can lead to further tissue injury and damage. The antimicrobial activity may partly contribute to the wound healing effect by eliminating infection thus allowing the natural tissue repair processes to start. The antimicrobial activity may also play a useful role in accelerating the healing of old wounds by eradicating already established infection.[3,4] Another fungal infection that has been coming to light lately is the acute pseudo membranous candidiasis (commonly

known as thrush). At present, it is considered the most widespread form of oral candidiasis-as many as 5% of newborn infants, 5% of cancer patients, and 10% of all hospitalized and debilitated elderly patients will come down with the disease.[4]

Common synthetic drugs used for treatment Topical Antifungals (e.g. Ketoconazole, Miconazole, Clotrimazole, Selenium Sulfide, Zinc Pyrithone, Terbinafine, Ciclopirox, Allylamines) and Oral Antifungals (e.g. Ketoconazole, Fluconazole, Itraconazole). Both classes act by targeting a particular intermediate, but have various side effects which manifest on their long-term use. Thus, there is a need to develop an alternative therapy to combat these side effects. Reports have implicated herbal medicine as an alternative therapy to conventional therapy mentioned above. Herbal medicine is the oldest form of healthcare known to mankind and is useful for wide range of diseases with little or no side effects. Generally, an herbal formulation consists of various active compounds which act synergistically to give the desired effect. Many traditional Ayurvedic formulations are available throughout the traditional literature which proposes to have a particular therapeutic action, but lacks in the method of standardization to obtain consistent results.[5]

Cassia species have been reported to treat fungal infections. *C. alata, C. fistula*, and *C. tora* are recommended for primary health care in Thailand to treat ringworm and skin diseases. In Thai traditional medicine, laxative pills are obtained by boiling the ripe pod pulp of *C. fistula* with water. The leaves are also applied externally for skin eruption, ulcer, wound, eczema, and ringworm. Another work reported high antifungal activity of anthraquinone aglycones extracted from glycosidic fraction of Sennaalata leaves against clinical strains of dermatophytes.[6] In Ayurvedic literature, powder and ointment are prepared from dried fruits and seeds of karanj.[7] Oil of *Pongamia Pinnata* (Karanj oil) and leaves of *Cassia fistula* Linn both have reported antifungal and wound healing properties.[8-11] In the present work, leaves of *Cassia fistula* Linn and oil of *Pongamia Pinnata* (Karanj oil) have been used to formulate together for synergistic effect and achieve better patient compliance.

MATERIALS AND METHODS
Leaves of *Cassia fistula* Linn (Caeselpaniaceae) were collected from Botanical garden of AISSMS College of Pharmacy, Pune-01. Rhein and Karanjin were purchased from Yucca Enterprises Mumbai, India). Karanj oil was purchased from Ayurvedic pharmacy.

Collection of crude drug
The leaves of *Cassia fistula* Linn (Caeselpaniaceae) were collected from Botanical garden of AISSMS College of Pharmacy, Pune-01. The leaves were thoroughly washed and the foreign matter like twigs, flowers, unwanted material were removed by handpicking method and kept for shade drying.

Authentication of crude drug
A portion of collected leaves was dried and about 100 g of each drug was given for authentication at Botanical Survey of India, Pune-01. The authentication was done by taking into consideration the taxonomical properties of crude drug.

Preparation of extract
Leaves were washed; shade dried and powdered in mixer grinder. 50 g of powder was dissolved in 400 mL of methanol and kept for cold maceration for 7 d. Liquid extract was filtered through Whatmann filter paper. Methanol was evaporated by Rotary evaporator. Extract was prepared and stored in refrigerator at 4°C. Two batches were prepared and % yield was found to be 47.72 and 16.02.

Development and formulation of cream
Drug excipient compatibility
IR studies were carried out on the formulation excipients such as tween 80 and span 20 with karanj oil and *Cassia fistula* extract to check for any incompatibilities. These samples were stored at 40^{0}C for 2 weeks and subjected to FTIR analysis using spectrophotometer between 400cm^{-1} to 4000 cm^{-1}.
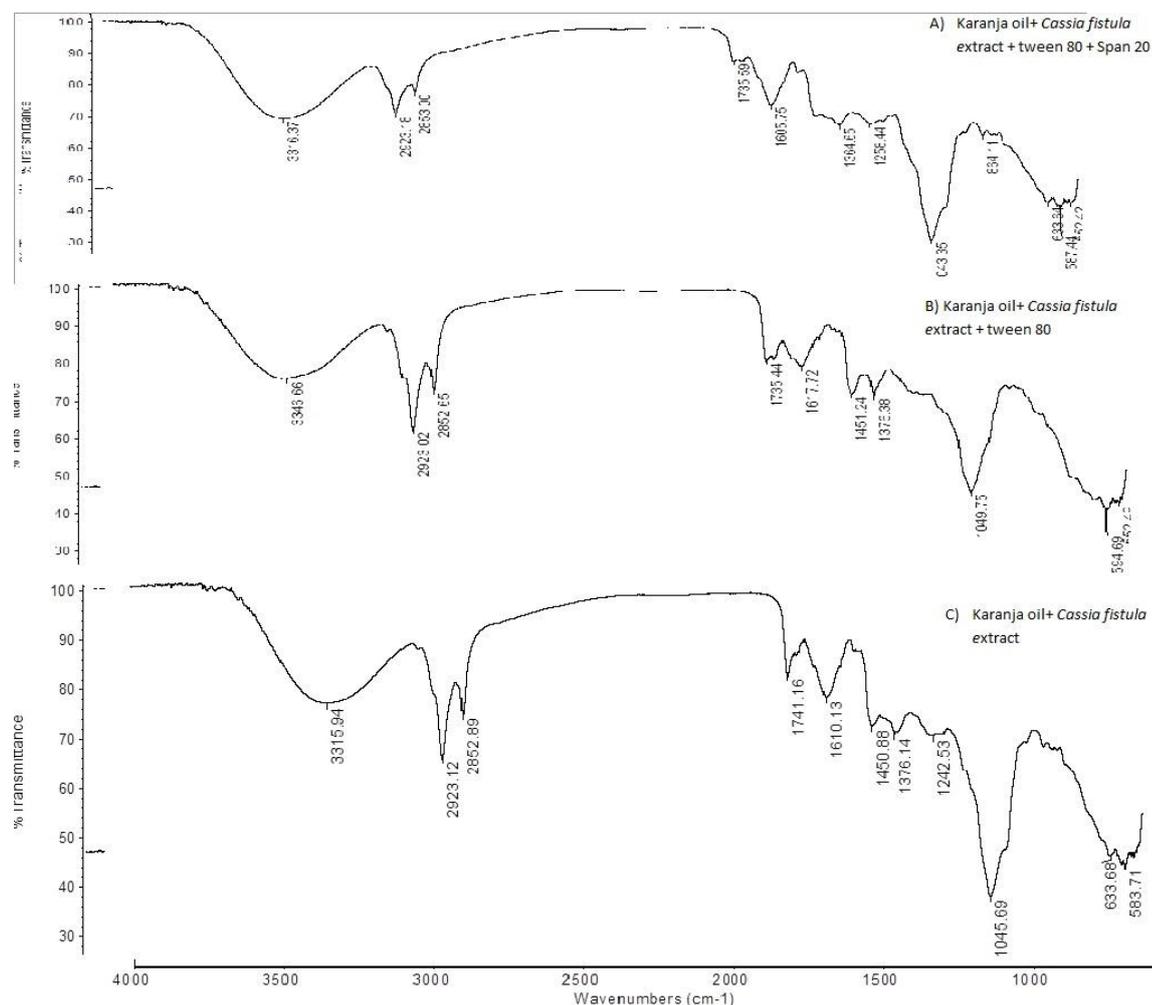
Figure 1: IR spectrum for drug excipient compatibility.

Selection of cream formula
Cream formula was selected from literature. [12] The list of ingredients and quantity used to prepare the antifungal and wound healing cream is depicted in Table 1.

Table 1: Formula for cream containing *Cassia fistula* extract and karanj oil.

Sr. no	Ingredients	Quantities
1	Liquid Paraffin	1.6 g
2	Stearic acid	1 g
3	Glyceryl monostearate	0.3 g
4	Span 20	1 mL
5	Karanj oil	2 mL
6	Glycerine	0.4 mL
7	Tween 80	1 mL
8	*Cassia fistula* extract	1 mL
9	q.s. water	20 mL

Selection of experimental design
The cream formula was optimized by employing full factorial design. Design-Expert (Version 10.4.0; Stat-Ease Inc., Minneapolis, Minnesota, USA) was used for mathematical modeling and assessment of the responses. A 3^2 factorial design was chosen to select a formula

which will provide optimum viscosity, hardness and drug release.

Table 2: The factor combination and response chosen for factorial design.

Variables		Levels		
A	Stearic acid	+1	0	-1
		3 gm	2 gm	1gm
B	Tween 80: Span 20	6%	4%	2%
Responses		**Goals**	**Acceptance range**	
X_1	Spreadability	Optimum	<15 g	
X_2	Viscosity	Optimum	<700 Cp	
X_3	Drug diffusion	Maximum	91-101%	

Preparation of cream
Oil phase was obtained by melting stearic acid, liquid paraffin, glycerylmonostearate, span 20 and karanj oil at 70°C separately. Aqueous phase was obtained by heating *Cassia fistula* extract, tween 80, glycerin and water at 70°C separately. The aqueous phase was slowly added to the oil phase at 40° C with constant stirring using mechanical stirrer at 100 rpm (Remi motor RQT-127

HP1/8) for 30 min till the uniform distribution of the ingredients was achieved.

Evaluation of cream
a) **Determination of pH:** Accurately weighed quantity of cream was dispersed in water to prepare 1 % w/v concentration. Calibrated pH meter (Make: I, DELUXE-101) was used to determine the pH.
b) **Determination of spreadability:** The spreadability of cream was determined by using Brookfield Texture Analyzer (CT 100). Cream (20 g) was taken in the cup of the texture analyzer, previously aligned with the probe. The hardness values obtained were recorded.
c) **Determination of viscosity:** (Brookfield digital viscometer RVDV Pro) equipped with ULE adapter was used. The spindle (S06) was rotated at 50 rpm. Samples of the cream were allowed to settle over 30 min at the temperature (25 ± 10ºC) before the measurements were taken. Viscosity was reported in (Cp).
d) **Determination of globule diameter:** The globule diameter of 1% dispersion of cream in water was measured by using Malvern Zetasizer ZS 90 UK.
e) **In vitro drug release:** The release of drug from the cream was determined using Franz diffusion cell apparatus for 6 h. The receptor medium was phosphate buffer pH 6.8, maintained at 37°C. The membrane filter (cellulose acetate) pore size 0.45 µ was soaked in phosphate buffer pH 6.8 for 12 h and mounted between the donor and receptor compartment. The cream was placed on receptor compartment and both the compartments were clamped together. The phosphate buffer pH 6.8 in the receptor compartment (8 mL) was stirred using magnetic stirrer 60 rpm. The samples (1 mL) were withdrawn at different time intervals and replaced

with an equal volume of buffer. The samples were analyzed spectrophotometrically at 260 nm and 257 nm, respectively. The % cumulative drug releases were calculated.

RESULT AND DISCUSSION
Selection of drugs
The drugs used for the study were the leaves of *Cassia fistula* and Karanj oil as their antifungal and wound healing activity is well established in traditional literature. *Cassia fistula* is rich in anthraquinones and phenols. Activity of the plant is associated with the presence of chemical components such as phenols, tannis, saponins, alkaloids, steroids, flavonoids and carbohydrates. Traditionally this plant is effective in treating skin infections. Karanj seed oil contains Karanjin, pongamol, pongapin and Kanjone which is responsible for antifungal and wound healing activity. The phytoconstituents considered for the present study were Rhein and Karanjin since their antifungal and wound healing activity is well documented and constituted major portion of the phytoconstituents. Karanj oil was chosen as base since it is widely used in Ayurvedic formulations apparently due to its permeation enhancer properties.

Evaluation of cream
The pH of all batches was in the range of 6.5-7 and so, it was concluded that the cream is non-irritant to the skin *in vitro*. The hardness of the creams (F1-F9) was between 21.89 g-44.88 g. The viscosity of formulations (F1-F9) was in range of 8400-10872 cps. The values for globule diameter of formulations F1-F9 were found in the range of 466.5-618.9 nm. As tween80: span 20 concentration increases drug release increases and as stearic acid concentration decreases drug release decreases.

Table 3: Evaluation parameter of formulation.

S. No.	Formulation code (X1g, X2 %)	Spreadability (Hardness)	Viscosity (Cps)	pH	Globule diameter (nm)	Drug release (%)	
						Karanjin	Rhein
1	B1 (1.0, 2.0)	21.89	7510	6.87	732.9	25.12	22.77
2	B2 (2.0, 2.0)	41.6	8400	6.65	475.0	24.88	30.12
3	F3 (3.0, 2.0)	44.88	10872	6.55	466.5	11.39	20.17
4	F4 (1.0, 4.0)	29.87	6983	7.00	481.0	36.53	20.62
5	F5 (2.0, 4.0)	37.4	8109	6.97	492.3	45.84	35.22
6	F6 (3.0, 4.0)	41.98	10387	6.25	618.9	24.98	20.48
7	F7 (1.0, 6.0)	38.9	7259	6.95	702.0	31.89	23.12
8	F8 (2.0, 6.0)	41.76	8523	6.38	518.3	35.22	32.18
9	F9 (3.0, 6.0)	28.95	9172	6.87	789.2	22.53	20.44

Design of Experiments
3^2 full factorial design was chosen as the optimization design since it allows the comparison of all the levels and factors to obtain significant results. The responses such as viscosity, spreadability, in vitro drug release were chosen for optimization which depended mainly on quantity of lipid and the surfactant hence these were

selected as factors. The three levels give good idea about interaction between factors if any.

Table 4: Design and evaluation of herbal cream.

Formulation code	Factor A: Stearic acid	Factor B: Tween80:span20	Response 1: Viscosity (Cp)	Response2: Spreadability (g)	Response 3: Drug release 1 and 2 (%)	
					Karanjin	Rhein
B1	1	2	7510	21.89	25.12	22.77
B2	2	2	8400	41.6	24.88	30.12
B3	3	2	10872	44.88	11.39	20.17
B4	1	4	6983	29.87	36.53	20.62
B5	2	4	8109	37.4	45.84	35.22
B6	3	4	10387	41.98	24.98	20.48
B7	1	6	7259	38.9	31.89	23.15
B8	2	6	8523	41.76	35.22	32.18
B9	3	6	9172	28.95	22.53	20.44
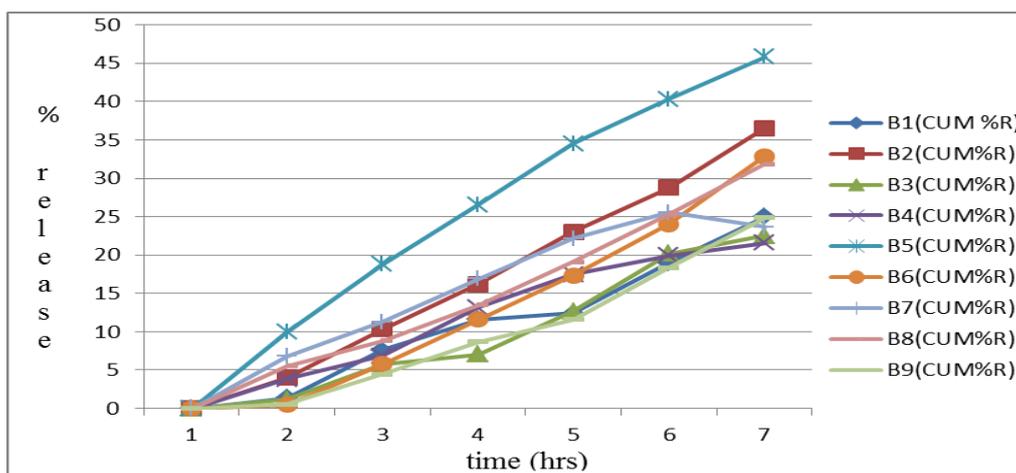
Figure 2: % in vitro drug release (karanjin) from optimization batches

Figure 3 depicts response surface curve for viscosity of cream.

Equation 1: $Y1= 6456.2+758.3X1-49.41X2-181.12X1X2+353.16X1^2 + 32.41X2^2$ ……. (1)

The model terms for the cream viscosity was found to be significant with high value of R^2 0.9575 which indicates the adequate to a quadratic model. Values of prob F was less than 0.5 indicated that the model terms were significant. The predicted R^2 of 0.5088 is in reasonable agreement with the adjusted R^2 0.8866; i.e. the difference is more than 0.2. Adequate precision measures the signal to noise ratio. A ratio greater than 4 is desirable. From Equation 1 and 3D response curve it was deduced that as the concentration of stearic acid increases the viscosity increases as the cream thickens due to increasing amount of thickening agent and tween 80:span 20 has no significant effect on viscosity.
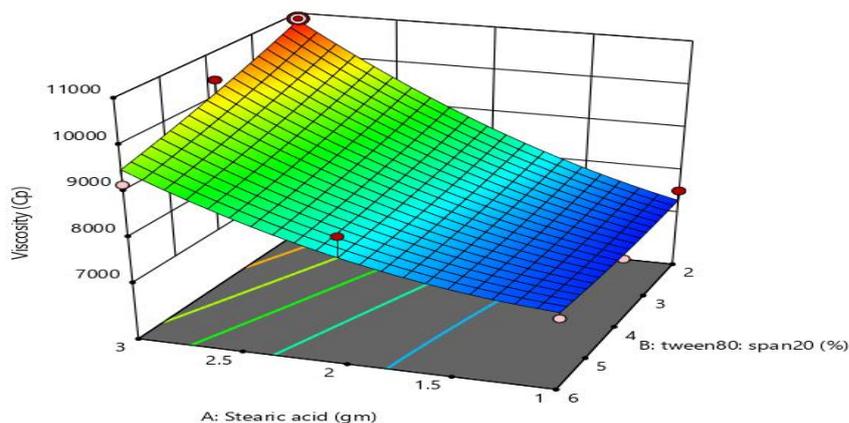
Figure 3: Response surface depicting effect of tween 80: span 20 and stearic acid on viscosity

Figure 4 depicts response surface curve for hardness of cream.

Equation 2: $Y2 = -25.13 + 44.02X1 + 8.51X2 - 4.11X1X2 - 5.8X1^2 - 0.02X2^2$ ……… (2)

The model terms for the cream spreadability was found to be significant with value of R^2 0.9378 which indicates adequate to a quadratic model. Values of prob F was less than 0.5 indicated that the model terms were significant. The predicted R^2 of 0.4369 is in reasonable agreement with the adjusted R^2 is 0.8341 i.e. the difference is more than 0.2. Adequate precision measures the signal to noise ratio. A ratio greater than 4 is desirable. From Equation 2 and 3D response curve it can be deduced that as the concentration of stearic acid increases hardness also increases and as the concentration of tween 80: span 20 increases hardness also increases.
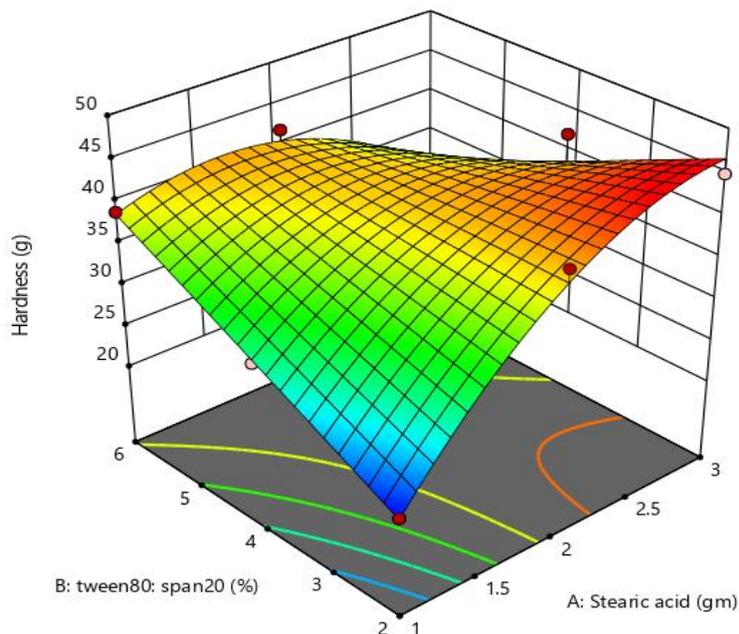
Figure 4: Response surface depicting effect of tween 80: span 20 and stearic acid on Hardness.

Figure 5 indicates response surface curve for drug release of cream.

Equation 3: $Y3 = 18.48 - 17.07X1 + 20.73X2 + 1.53X1X2 + 0.58X1^2 - 2.90X2^2$ ……… (3)

The model terms for the cream drug release of karanjinwas found to be significant with high value of R^2 0.9651 which indicates the adequate to a quadratic model. Values of prob F was less than 0.5 indicated that the model terms were significant. The predicted R^2 of 0.7365 is in reasonable agreement with the adjusted R^2 0.9069 i.e. the difference is less than 0.2. Adequate precision measures the signal to noise ratio. A ratio greater than 4 is desirable. From Equation 3 and 3D response curve it was found that Tween 80: span 20 has positive effect on drug release. As the concentration of tween 80: span 20 increases drug release also increases and as the concentration of stearic acid decreases drug release increases.
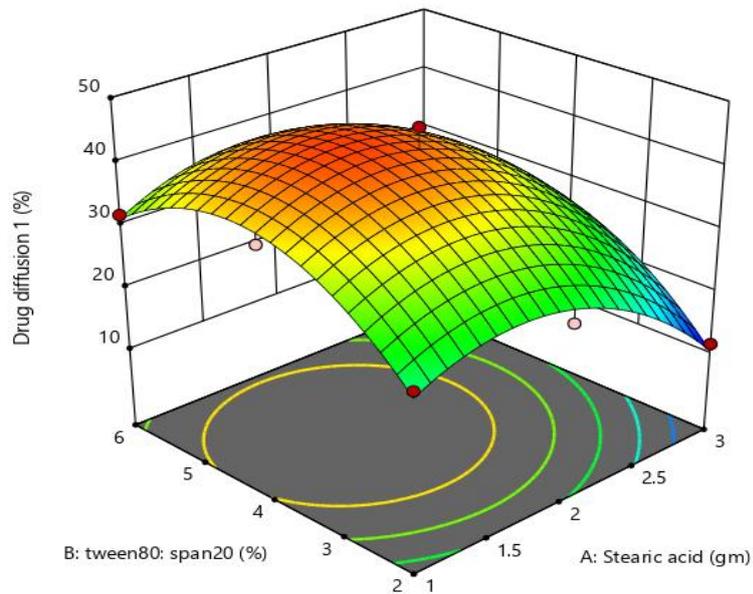
Figure 5: Response surface depicting effect of tween 80: span 20 and stearic acid on drug release (Karanjin).

Figure 6 represents response surface curve for drug release of cream.

Equation: $Y4 = -13.74 + 44.08X1 + 1.52X2 - 0.013X1X2 - 11.235X1^2 - 0.158X2^2$ ………. (4)

The model terms for the cream drug release 2 was found to be significant with high value of R^2 0.9456 which indicates the adequate to a quadratic model. Values of prob F was less than 0.5 indicated that the model terms were significant. The predicted R^2 of 0.5418 is in reasonable agreement with the adjusted R^2 0.8551 i.e. the difference is less than 0.2. Adequate precision measures the signal to noise ratio. A ratio greater than 4 is desirable. From Equation 4 and 3D response curve it was found that as the concentration of stearic acid increases drug release also increases and tween 80: span 20 has no significant effect on drug release.
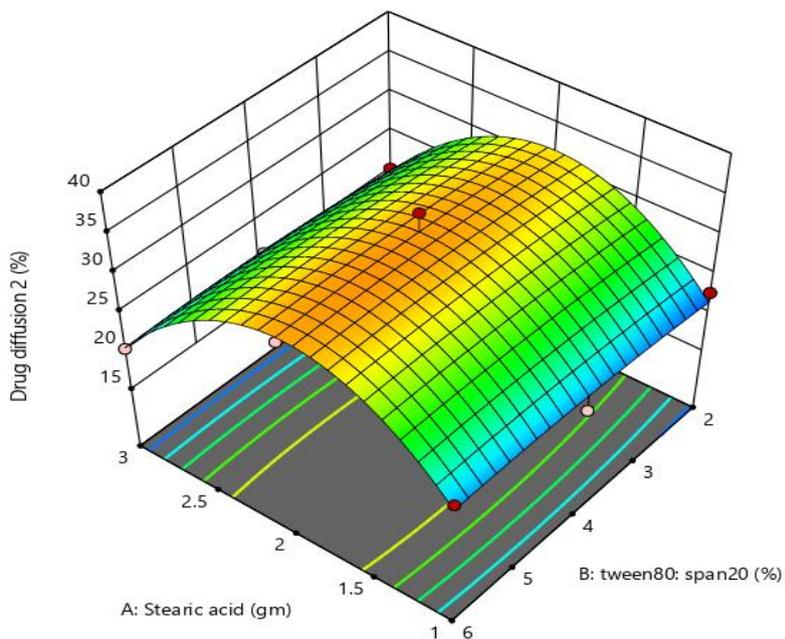
Figure 6: Response surface depicting effect of tween 80: span 20 and stearic acid on drug release (Rhein).

CONCLUSION

Batch 5 formulation or cream was found to be best and satisfactory compared to all other formulations. It had light green appearance, gave a cool and smooth feel on application. The pH of the formulation was found to be 6.97 which are good for skin. The creams also showed good Hardness (30 g.cm/sec) when measured using a texture analyzer. After application of the cream the type of smear formed on the skin was found to be non-greasy and easily removed on washing with tap water. The viscosity of the creams was found to be 7168.22 cps, with 50 rpm, which indicates that the prepared cream was easily spreadable with small amount of shear. The maximum drug release occurred through drug present in oil phase i.e. karanj oil as it is reported in literature that maximum drug release occurs through oil phase. Hence from all the results, it can be concluded that formulation and optimization of cream was successfully done.

ACKNOWLEDGEMENT

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