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Research Article

Transdermal Delivery of *Nigella sativa* Oil for Topical Application in 1-Chloro-2, 4-Dinitrobenzene- Induced Atopic Dermatitis in Rats

Ruchi Chawla*, Amit Chauhan

Department of Pharmaceutics, Indian Institute of Technology - Banaras Hindu University, Varanasi -221005 (U.P.) India

Correspondence: rchawla.phe@iitbhu.ac.in (Tel.+91 9336077707)

Abstract

Atopic dermatitis (AD), a chonic inflammatory disease, is associated with pruritic and relapsing skin lesions. Steroids, which form the mainstay of treatment of AD, generally produce side effects, which can be avoided by use of herbal formulations. Oil of *Nigella sativa* (NS) seeds contains thymoquinone, which has anti-inflammatory properties. With this objective, cream of *Nigella sativa* oil for transdermal use in AD was developed and evaluated. Using phase inversion temperature method, oil-in-water emulsion based cream was prepared and the optimized cream exhibited pH of 6.5 and viscosity of -2741.96 g-sec. Dermatitis was induced in rats using 1-Chloro-2, 4-dinitrobenzene and the therapeutic efficacy of NS oil cream was compared with that of betamethasone valerate (BMV), a steroidal cream. Histological analyses of skin sections showed decrease in both, epidermal thickening as well as infiltration of inflammatory cells on application of NS oil cream. Comparable alleviation of dermatitis symptoms was observed by application of NS oil cream and BMV cream. So, cream of *Nigella sativa* oil might be considered as a potential substitute for treatment of AD.

Keywords: *Nigella sativa*, atopic dermatitis, cream, topical application, 1-Chloro-2, 4-dinitrobenzene.

Introduction

Atopic dermatitis (AD) is a chonic inflammatory skin disease, the pathogenesis of which is correlated with specific immune and inflammatory mechanisms involving Type 1/ type 2 helper (T_H1/T_H2) cell dysregulation, Immunoglobulin E (IgE) production, mast cell hyperactivity, and dendritic cell signaling, which contribute to the occurrence of chonic, pruritic, inflammatory dermatitis that characterize AD [1,2]. Biologically active concentrations of leukotriene B_4 (LT B_4), a 5-lipoxygenase metabolite of arachidonic acid, along with, higher levels of cyclooxygenase product, prostaglandin E_2 (PGE_2) have been observed in atopic dermatitis [3,4].

Nigella sativa (Black seed), an annual flowering plant of botanical family ranunculaceae, is a commonly used ingredient of edible preparations. Black seeds known as 'seeds of blessing' have been used as a protective and curative for centuries in Greco-Arabic, Islamic, Indian and Chinese traditional medicine for the treatment of bronchial asthma, headache, dysentery, etc.[5]. The seeds or its extracts have been used widely for their therapeutic properties such as immune stimulation and modulation [6], anti-inflammatory [7, 8], anti-tumor [9], and anti-oxidant effects [10]. Most of these pharmacological effects are attributed to the presence of thymoquinone (TQ), one of the active constituents of volatile oil extracted from the seeds of *Nigella sativa* [11]. TQ inhibits generation of inflammatory mediators, thomboxane B_2 and leukotriene B_4, by targeting cyclooxygenase and 5-lipoxygenase pathways of arachidonic acid metabolism [7]. Usefulness of *Nigella sativa* seeds and its extracts has been reported in a variety of dermatological conditions like psoriasis, acne vulgaris, vitiligo, skin cancer, and microbial, viral, fungal and parasitic infections. Treatment of neonates with staphylococcal pustular skin infections with *Nigella sativa* extract showed results similar to topical mupirocin with no side effects [12]. Intra-peritoneal administration of NS oil in mice infected with murine cytomegalovirus for 10 days showed undetectable levels of virus in liver and spleen, while the same could be detected in control animals. The experimental outcome was correlated to increased levels of CD4+ T cells and INF-gamma [13]. Wound healing property of *Nigella sativa* seed and its oil was reported as early as 1995 by Ahmed et al., (1995) [14]. Topical application of ether extract of NS seed onto staphylococcal-infected mice skin evidently enhanced healing by reducing total and absolute differential WBC counts, local infection and inflammation, bacterial expansion and tissue impairment [15]. Twice daily topical application of NS oil in lesions of vitiligo patients for 6 months significantly decreased vitiligo area with no significant side effects [16]. A clinical study on patients with allergic dermatitis, showed effectiveness of *Nigella sativa* oil in decreasing levels of IgE, eosinophil count, endogenous cortisol in plasma and urine [17]. Also, studies have shown that *Nigella sativa* down-regulates release of histamine from mast cells [18].

United States Pharmacopeia defines cream as “a semisolid emulsion that contains more than 20% of aqueous phase and/or less than 50% of oleaginous phase for external application to the body skin”. Non-occlusive and water-washable creams (oil-in-water emulsion type) are preferred by patients as they provide a pleasant skin feel and are easily washed from skin surface because of their non greasy texture [19]. The properties of a formulation are influenced by compositional factors (variants) such as concentration of emulsifier, stabilizer, buffering agents, etc. used in the preparation of a formulation. Experimental design approach (DoE) helps to evaluate simultaneous or combined effect of formulation factors (independent variables) on the formulation properties (response) such as particle size, viscosity, drug release properties, etc. Using DoE, experimental trials can be done by holding certain factors constant and altering the levels of other variables i.e. changing factor levels simultaneously, which is not possible with One–Factor–at–a–Time (OFAT) approach [20].

In the present study, in order to explore dermatological application of *Nigella sativa* oil, we have prepared cream of NS oil and evaluated its therapeutic potential in alleviating dermatitis symptoms in 1-Chloro-2,4-dinitrobenzene (CDNB)- induced atopic dermatitis in rats.

Materials and Methods

Materials

Seeds of *Nigella sativa* (NS) were procured from a local market in Varanasi. The seeds were cleaned and air dried in shade for a few days and the samples were certified by Department of Botany (BHU), Varanasi, India (Voucher specimen no. Ranun. 2014/1).

Oil was extracted from *Nigella sativa* seeds by cold press method using screw press expelling machine. Thymoquinone (99% purity) was purchased from Sigma Chemicals (Saint Louis, MO). Stearic acid, triethanolamine, disodium EDTA, glycerol, propane-1,2-diol, sodium metabisulphite, and cetostearyl alcohol were purchased from S.D. Fine Chemicals (Mumbai, India). All solvents used for analysis were of HPLC grade and were purchased from Merck Ltd., (Mumbai, India).

Methods

Assay of NS oil

Thymoquinone was chosen as the marker compound for standardization of NS oil. Reverse phase HPLC method was used for estimation of thymoquinone content (Adept CECIL, UK). Pure thymoquinone was dissolved in methanol to prepare a standard stock solution of 100 µg /ml. For calibration, working stock solutions in the concentration range of 1-50 µg/ml were prepared by dilution with mobile phase. Water and methanol (40:60) were used as mobile phase for analysis by isocratic elution. Thymoquinone content in oil was analyzed by diluting 1ml of NS oil to volume with mobile phase in a 20 ml volumetric flask. 20 µL aliquot of sample solutions were injected into HPLC and analyzed on Waters XTerra RP18, 5μm, 250mm $\times$ 4.6mm i.d column at a flow rate of 1.5 ml/min and detection wavelength of 254 nm.

Preparation of cream

The ingredients used for the preparation of cream have been mentioned in Table 1.

Table 1: Prototype and composition of *Nigella sativa* oil cream

Ingredients (%w/v)	Properties	Composition
Nigella sativa seed oil	Wound healing	4.0
Stearic acid	Emulsifying agent, forms two dimensional crystal network, emollient, skin-softening properties	A+
Cetostearyl Alcohol	Emulsion stabilizer, opacifying agent, foam boosting surfactant, aqueous and non aqueous viscosity-increasing agent.	2.5
	Water soluble	
Triethonalamine	Emulsifier and surfactant, neutralizes fatty acids	B*
Glycerine	Humectant and plasticizer	4
Propylene glycol	Humectant and plasticizer	8
Sodium metabisulfite	Antioxidant	0.1
Disodium EDTA	Chelating agent	0.1
Purified Water		q.s.

*A and *B will be varied as per the matrix generated for face centered experimental design (Table 2)

The selection of ingredients was based on their properties to produce an optimal oil-in-water emulsion based cream. Phase inversion temperature method with modifications was used for the preparation of cream [21]. The heuristics of sequence of mixing of ingredients were based on the basic phenomenon of emulsification. The oily phase consisting of oil soluble ingredients was prepared by stirring (200 rpm) cetostearyl alcohol and stearic acid under mild heating condition using a hot magnetic plate stirrer (Magnetic Stirrer IKA), thereafter maintaining the temperature at 65 °C. The aqueous phase was prepared by mixing water soluble ingredients (disodium EDTA, glycerine, sodium metabisulfite, propylene glycol and triethanolamine) under gentle heating and stirring. Temperature of aqueous phase was also maintained at 65 °C. Oil of Nigella sativa was added gently under stirring to the oily phase, immediately before mixing of the two phases. Emulsion was prepared by slowly adding aqueous phase along the walls of container under stirring (200 rpm) and gentle heating. For optimal emulsification, the emulsion was stirred at 800 rpm for 45 min at 65 °C and left undisturbed for cooling. Also, blank cream (containing no NS oil) was prepared following the same procedure.

Design of experiment for formulation of cream

A 2-factor, 3-level face centered design was employed for studying the effect of emulsifier concentration on characteristics of cream. The experimental design consisted of two groups of design points: two factorial level design points fixed at +1 and −1; and a centre level design point fixed at 0. Two independent variables (factors): concentration of stearic acid (A); and concentration of triethonalamine (B) were evaluated at factor levels -1, 0, +1 for their effect on viscosity index; and cumulative percentage drug release, which were studied as response. All other formulation ingredients and processing variables were kept invariant throughout the study. Experimental matrix and experimental plan, along with constraints applied have been shown in Table 2 and Table 3. All experiments were performed in randomized manner to eliminate possible sources of bias.

Table 2: Levels and constraints employed for face centered experimental design

Factors (variable)	-1	0	1
A = Stearic acid concentration (%w/w)	12	13.5	15
B=Triethanolamine concentration (%w/w)	1.0	1.5	2.0
Response	**Constraints**		
Index of viscosity	Minimize		
Cumulative % drug release	Maximize		

Table 3: Experimental matrix with coded and actual value of variables

Formulation batches	Coded factors		Actual values of factors	
	A	B	Stearic Acid concentration (%w/w)	Triethanolamine concentration (%w/w)
F1	-1	-1	12%	1%
F2	-1	-1	12%	1.5%
F3	-1	+1	12%	2%
F4	0	0	13.5%	1%

Formulation batches	Coded factors		Actual values of factors	
	A	B	Stearic Acid concentration (%w/w)	Triethanolamine concentration (%w/w)
F5	0	0	13.5%	1.5%
F6	0	+1	13.5%	2%
F7	+1	-1	15%	1%
F8	+1	0	15%	1.5%
F9	+1	+1	15%	2%

The experimental design facilitates estimation of constant, linear terms, interactions between variables and quadratic terms, according to the following model (usually, not taking into account the interactions among more than two factors) [22]:

$$Y = b_0 + b_1A + b_2B + b_{12}AB + b_{11}A^2 + b_{22}B^2$$

where Y indicates measured response, b_0 intercept, b_1 and b_2 linear coefficients, b_{11} and b_{22} quadratic coefficients, b_{12} interaction coefficient, and A and B are independent variables, and polynomial terms (A^2 and B^2) analyze non-linearity. Coefficient of a factor (variable) with a positive value signifies variation of response directly with increase in the value of variable. Conversely, response decreases with increase in the value of a variable when the coefficient is negative. The statistical analysis of data was performed using Design Expert 9.0.7.1 software. A good fit experimental model exhibits a low value of predicted residual sum of square (PRESS) and a high (closer to 100%) R^2 value. Individual as well as interactive effects of factors on response were analyzed by statistical analysis of variance (ANOVA). p-value of less than 0.05 was considered as statistically significant.

Characterization of cream formulation

Sensorial evaluation

The prepared cream was evaluated for its aesthetic properties, physical appearance, color, odor, and texture. Further, batches of cream were evaluated for their consistency by examining softness, greasiness, and stickiness. Ideally, non-greasy, non-sticky, consistent, and spreadable creams are preferred by users.

Evaluation of emulsion type

In order to ascertain the emulsion type, dye test was performed. Water soluble dye (amaranth) was added to cream, spread uniformly on a glass slide and observed under a microscope. For an oil-in water emulsion, continuous red phase will be observed; however, in a water-in-oil emulsion red colored spots will be seen against a dark background.

Evaluation of cream pH

50 gm of cream was weighed, diluted and mixed with distilled water. pH of cream was measured using a digital pH meter. All measurements were carried out in triplicate.

Texture profile analysis

Texture profile analysis (TPA) was performed using a Stable Micro Systems Texture Analyzer (TA-XT2® Texture Analyzer, Surrey, UK). The cylindrical analytical probe of 3 cm diameter was compressed into each sample at a defined rate (pre-test speed: 3.0 mm sec^{-1}; test speed: 1.0 mm sec^{-1}; and post-test speed: 1.0 mm sec^{-1}) to a distance of 10 mm, applying a trigger force of 5 gm. During analysis the temperature was maintained at 37 ºC and all analyses were performed in triplicate. From the resultant force–time plots, cream characteristics such as firmness, consistency, cohesiveness and index of viscosity were assessed.

***In vitro* drug release studies**

In vitro release studies were carried out in glass diffusion cell. Cellulose membrane (mol. wt. 12-14000 Da, Himedia, India) hydrated overnight in phosphate buffer (pH 6.8), was firmly placed between the donor and receptor compartments of diffusion cell. The receptor compartment was filled with 100 ml phosphate buffer, pH 6.8, used as dissolution media, and maintained at 37±1 ºC with continuous stirring at 50 rev/min on a hot plate magnetic stirrer. After equilibration for half an hour, cream sample was applied on the membrane in donor compartment. Aliquots (5 ml) were withdrawn from receptor compartment at regular intervals and replaced with equal volume of fresh media. The amount of thymoquinone released in the samples was analyzed for at 254 nm by UV-Vis spectrophotometer. The drug release kinetics were studied by applying various kinetic models: Zero order, first order, Higuchi and Peppas - Korsemeyer and based on correlation coefficient (R^2) values drug release pattern was predicted.

***In vitro* occlusion test**

Three pre-weighed beakers of 50 ml capacity were filled with 25 ml of double distilled water, covered with standard laboratory filter papers and sealed firmly. 250 gm of each of blank cream and NS oil cream were applied evenly on the surface of filter papers of two beakers. The beakers were stored at 32 ºC and 60±5% RH for 12 h, after which individual weights of beakers were recorded. Beaker with no cream sample served as comparative standard (control) for calculation of percent solvent loss (F), which was calculated as:

$$F = (A-B)*100/A$$

where A is solvent loss without sample and B is solvent loss with sample.

Stability studies

Stability testing of prepared formulations was carried at room temperature and 40 ± 2 ºC/ 75 ± 5%RH for 90 days. The formulations were evaluated for appearance, pH, consistency, removal and type of smear produced by the cream.

Animal experiments

Female rats weighing 160 ± 20 g and 6-8 weeks old were procured from Central Animal House Facility, Banaras Hindu University and were housed in plastic cages conditioned at 20 ± 2 °C and 50%–60% RH with alternate 12 h dark and light cycle. The animals were provided free access to pelleted food and distilled water *ad libitum* for 1 week prior to the commencement of study. General and environmental conditions were strictly monitored. Female rats were used for the study, as aggressive behavior of male animals leads to trauma of ears. All experimental protocols were approved by Animal ethical committee, Institute of Medical Sciences, BHU.

Skin Irritation studies

Induction of Irritation

Skin irritation studies were performed by the method described elsewhere with some modifications [23]. The animals, divided into two groups (n=9), were lightly anaesthetized with ether and ears were shaved using a sterile surgical blade. Approximately, 100 mg blank cream was applied to dorsal portion of right ear, and the left ear was marked as untreated control in Group I. Group II animals which were treated similarly with 100 mg of NS oil cream. Applications were made for four successive days and observations were made on fifth day of experiment.

Assessment and interpretation of irritation

Assessment of irritation was done by method described elsewhere [23]. Assessment was made for first four days, before daily application of cream was instituted, however, no application was made on the fifth day and observations were made 24 h after last (fourth) application of cream.

The severity of irritation was made based on assessment of symptoms as described on a numerical scale of 0 to 14 [no erythema (0), barely visible blood vessels (2), slight erythema over lower base of ear (4), generalized erythema with obvious blood vessels (6), pronounced erythema with internal hemorrhage and folding back of ear (8), marked erythema with possible frilling of ear margin (10), severe erythema with frilling, thickening and crusting of ear margins (12), obvious thickening of ear with necroses and crusting over whole ear surface (14)].

The daily differences between the scores of control and treated ears were summed to give an overall total for all the animals used in the experiment, which was then divided by nine (number of animals used in the trial), to give a mean score for each experimental group. The data obtained was interpreted for irritant nature of cream as: 0-6 (non-irritant to human skin), 7-10 (slightly irritant) and 11 or more (sufficiently irritant and unacceptable).

Induction of atopic eczema symptoms in rats and experimental design

Rats housed under pathogen-free conditions were acclimatized for two weeks, after which they were randomized into 4 groups (n= 9 per group): Group I- naive control (olive oil was used as vehicle); Group II- CDNB+ vehicle; Group III- CDNB+ NS oil cream; and Group IV- CDNB+ BMV cream (0.1% w/w). AD-like symptoms and skin lesions were induced by sensitization with CDNB. The backs of animals were shaved (approximately 2cm x 2cm) and a day after removal of hair, 100 μl of 1% CDNB dissolved in acetone: olive oil mixture (3:1 vol/vol) was applied on the dorsal skin (Day -4). The experimental scheme has been shown in Figure 1.

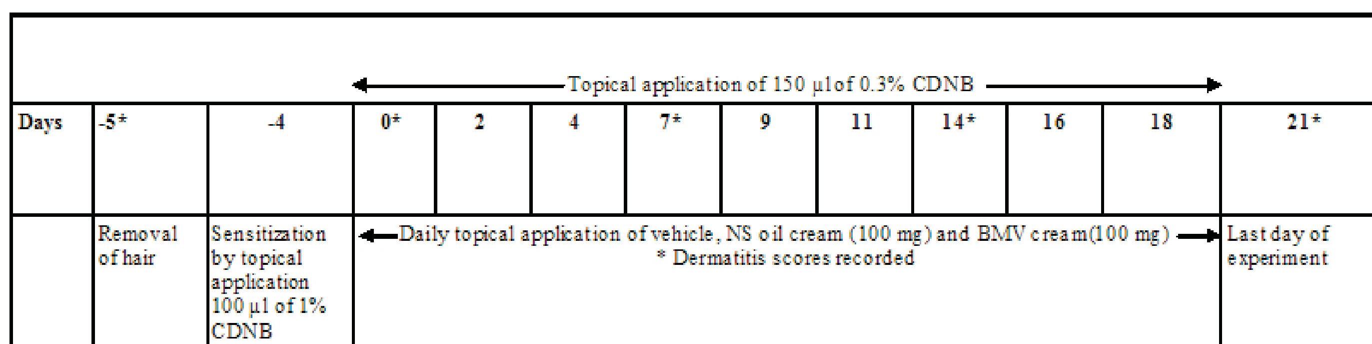

Figure 1: Protocol for induction of atopic dermatitis (1) Group I- naive control (vehicle), (2) Group II- CDNB +vehicle, (3) Group III- CDNB+NS oil cream (100 mg) and (4) Group IV- CDNB+ BMV (100mg). Rats were epicutaneously sensitized with 100μl of 1% CDNB to induce AD-like symptoms. Day –5: Dorsal hair removal, Day –4: Application of 100 μl 1% CDNB, Day 0-20: Topical application of 150 μl of 0.3% CDNB three times a week for three weeks; and NS oil cream or BMV cream were applied seven times a week for 3 weeks

Five days after removal of dorsal hair (day 0), 150 μl of 0.3% CDNB dissolved in acetone: olive oil mixture (3:1 vol/vol) was applied three times a week for three weeks to challenge the dorsal skin. NS oil cream (100mg/rat/day) or BMV cream (0.1% w/w) (100mg/rat/day) were applied daily on the dorsal skin surface for 3 weeks (day 0–20). The rats were anesthetized with 2% isoflurane at the end of experiment.

Examination of skin lesions

On the last day of experiment (day 21), rats were anesthetized with 2% isoflurane, and images of skin lesions of all treated groups were taken with a digital camera (Canon SX4 HS, Tokyo, Japan). In order to evaluate epidermal thickening, 4-mm-thick sections of dorsal skin of each animal were cut and prepared on the last day of experiment (day 21) by fixing with 10% neutral-buffered formalin, and subsequently preserved in paraffin. De-paraffinized skin sections were stained with hematoxylin and eosin (H and E) and tissue sections were examined at 100X magnification using Olympus AX70 light microscope (Tokyo, Japan).

Statistical analysis

Statistical analysis was performed using GraphPad InStat software (San Diego, California). All data were expressed as Mean ± SD (standard deviation). Data was analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test for comparing differences among multiple groups, $p<0.05$ was considered as significant.

Results and Discussion

HPLC analysis

HPLC chomatogram of thymoquinone in *Nigella sativa* oil has been shown in Figure 2.Thymoquinone was detected at 254 nm with a retention time of 7 min 30 sec. The standardized extract was found to contain 0.052% w/w of thymoquinone.

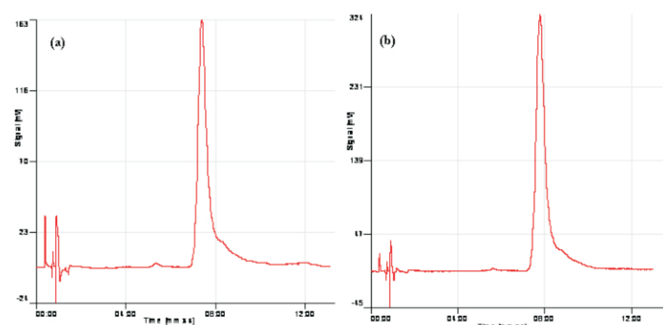

Figure 2: Typical HPLC chomatogram of (a) pure thymoquinone; and (b) Nigella sativa oil containing thymoquinone

Design of experiments for formulation of cream

A 2-factor, 3-level face centered design predicted second order experimental design and helped in understanding the main effect (influence of individual variables) and effects due to interaction of variables on the response functions (viscosity index and drug release).

The average viscosity index (Y_1) was observed in the range of -2741.963 gsec to -5675.680 gsec. The polynomial equation showing the effect of variables on viscosity index has been shown in equation 1:

Viscosity index $=4172.95 + 527.60*A - 809.80*B + 45.89*AB - 249.04*A^2 + 386.14*B^2$**... (1)**

Stearic acid (A) concentration exhibited a direct correlation with viscosity (positive sign of coefficient) ($p< 0.001$), however increase in triethonalamine concentration significantly decreased the viscosity index ($p< 0.05$). The response surface plots showing the effect of stearic acid and triethanolamine on viscosity index have been presented in Figure 3.

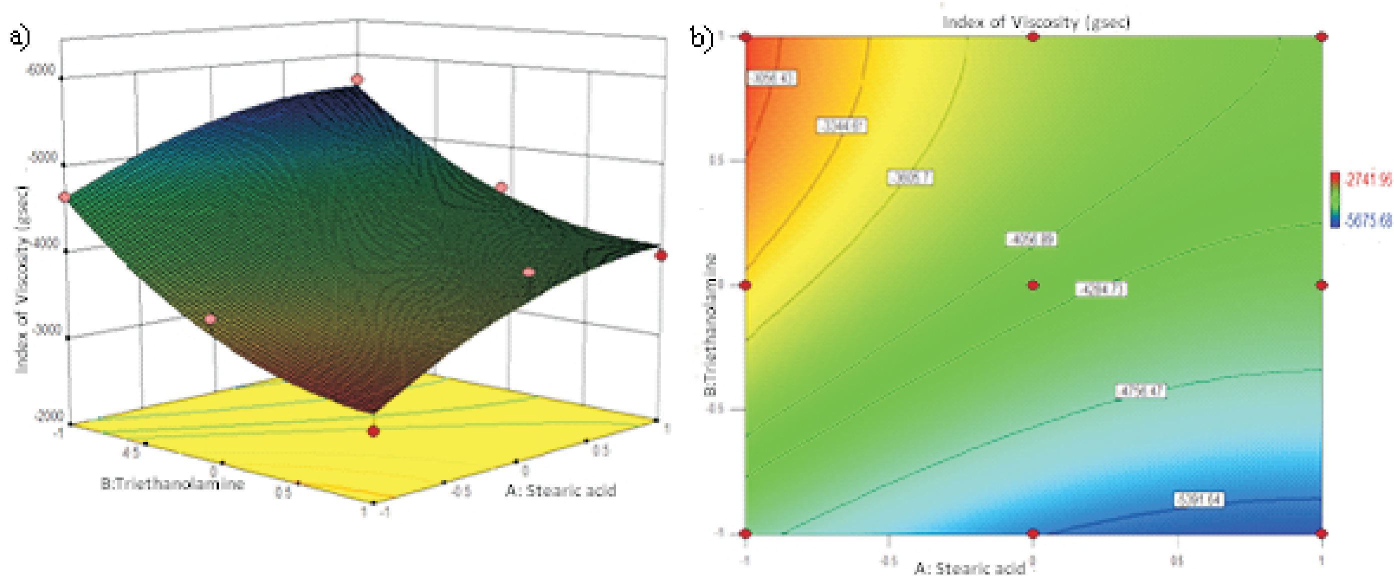

Figure 3: Response surface plots showing the effect of stearic acid (%w/w) and triethanolamine (%w/w) concentration on index of viscosity (g-sec) a) 3D plot; and (b) Contour plot

However, the combined effect of stearic acid and triethanolamine did not show a significant effect on the viscosity index. Stearic acid (solid at room temperature) is an efficient emulsifying agent, to which when triethanolamine (amine soap) is added, it gets neutralized, thereby resulting in decrease in viscosity of the formulation. The product triethanolamine stearate forms a homogeneous phase with the remaining fatty acid creating a liquid lamellar crystal which gradually transforms into a three-dimensional ordered structure [24]. The decrease in viscosity with increase in triethanolamine concentration could be attributed to neutralizing potential of triethanolamine.

The increase in triethanolamine concentration had a positive effect on cumulative % drug release (cum.% drug release) ($p<0.001$), however, a negative correlation of stearic acid concentration with cum.% drug release was observed ($p<0.001$) (Figure 4).

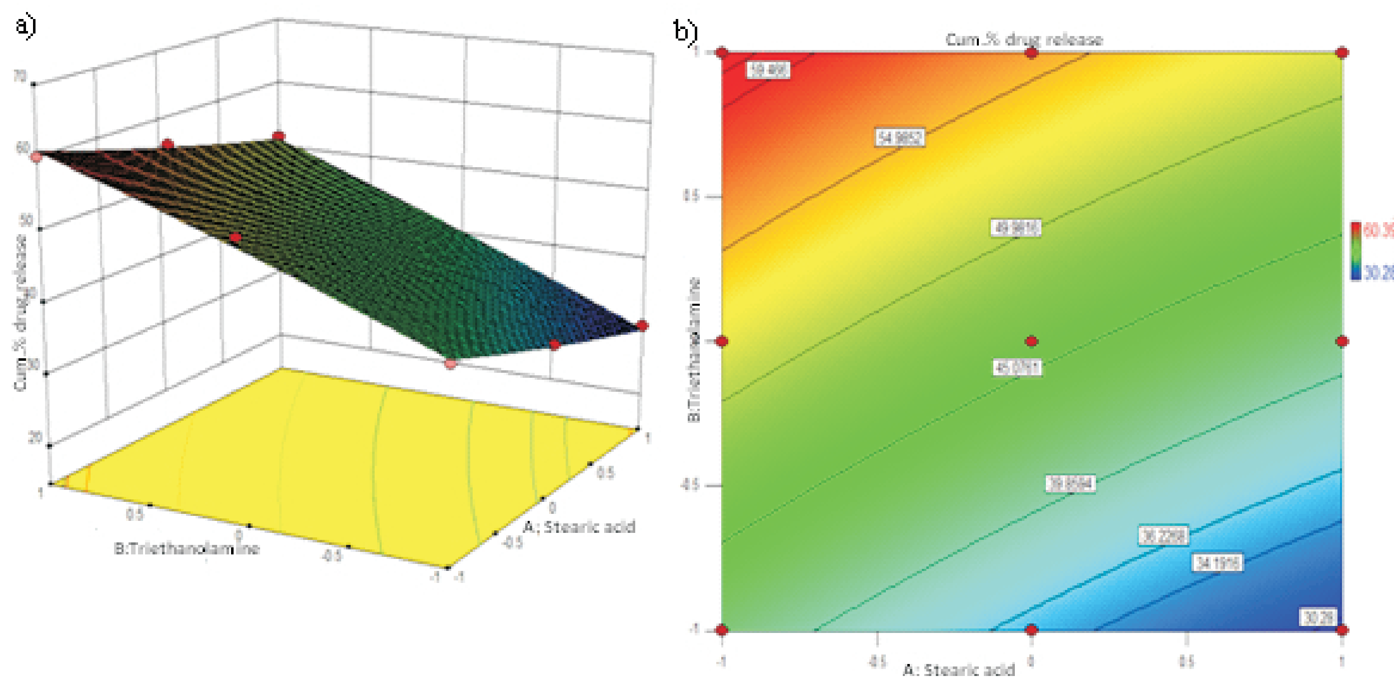

Figure 4: Response surface plots showing the effect of stearic acid (%w/w) and triethanolamine (%w/w) concentration on cumulative % drug release (Cum % drug release) a) 3D plot; and (b) Contour plot

Creams formulated with higher triethanolamine concentration were comparatively less viscous and facilitated the release of active constituent though the less rigid formulation network. The relationship between cumulative percent drug release and concentrations of stearic acid and triethanolamine was found to be:

Cum.% Drug Release = +46.11 - 5.44* A + 10.25* B + 0.62* AB + 0.45* A^2 - 0.48* B^2 ......(2)

The goodness of fit between predicted values and experimental values was substantiated by the value of correlation coefficient (R^2) of 99.35% and a low predicted residual sum of square (PRESS) value of 21.99.

The analysis of design matrix of experimental factors and corresponding responses as given in Table 4, showed that batch F_3 prepared using stearic acid concentration of 12 % w/w and triethonalamine concentration of 2 % w/w had a viscosity index - 2741.963 gsec and cum. % drug release of around 60%. Increase in stearic acid concentration (above 12%w/w), produced highly viscous and rigid network which stalled the diffusion of active constituents. There is a graded increase in cum.% drug release; and a corresponding decrease in viscosity of cream with increase in triethonalamine concentration. Increase in viscosity due to increase in stearic acid concentration (above 12% w/w) could not be profoundly decreased even at maximal triethonalamine concentration (2% w/w), probably because excess of stearic acid is left after neutralization reaction between triethanolamine and stearic acid, and this contributes to formation of a viscous network.

Table 4: Values of response (texture profile analysis data and cumulative % drug release) for the experimental batches of Nigella sativa oil cream and blank cream

Formulation Batches	Firmness (g)	Consistency (g-sec)	Cohesiveness (g)	Index of viscosity (g-sec)	Cumulative % drug release
F1	2867.841	3117.414	-2052.438	-4655.747	41.26
F2	1385.660	2187.658	-1336.594	-3563.472	53.36
F3	666.400	1056.256	-642.568	-2741.963	60.39
F4	2877.352	3175.087	-2075.535	-5277.132	35.48
F5	1421.342	2976.321	-1361.019	-3951.612	45.64
F6	1264.495	2107.137	-1041.182	-4062.371	56.24
F7	3115.621	3318.713	-2263.482	-5675.680	30.28
F8	2154.750	2988.321	-1782.372	-4505.680	40.22
F9	1269.264	2118.235	-1102.841	-3945.451	51.89
B3 ^	766.382	1912.327	-673.344	-2167.231	-

n=3; S.D and C.V not presented in table; ^ Blank cream

Characterization of cream formulation

Amongst the prepared nine batches of cream, batch F_3 exhibited optimal properties (response) and was considered for further characterization studies. The prepared cream was white in color and opaque with homogeneous appearance. The optimized batch possessed desired properties and was non-greasy and non-sticky favoring ease of application to skin surface. pH value of all the prepared batches was found to be in the range of 6.50 to 6.60.

Texture profile analysis

Force-time plots of NS oil cream (batch F_3) and blank cream (having no NS oil and composition similar to batch F_3) have been presented in Figure 5 (a and b).

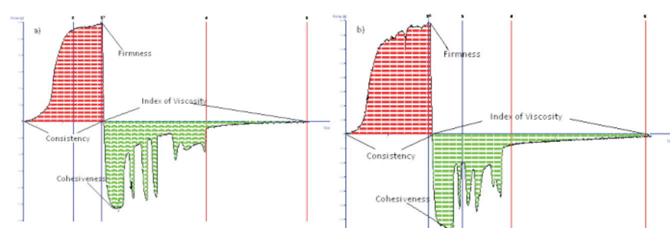

Figure 5: Force-time plot of a) NS oil cream (A3); & b) blank cream showing firmness, consistency, cohesiveness and index of viscosity

From the resultant force–time plots, the following mechanical parameters were studied: (i) Firmness - The peak force with which the probe moves into the sample (measured in the first quadrant of the force-time plot) denotes firmness. (ii) Consistency - It is the area covered by force-time plot in the first quadrant. (iii) Cohesiveness- It is the peak force in the fourth quadrant of force-time plot and is measured while the probe moves out from the sample. (iv) Index of Viscosity- It is the area covered by force-time plot in the fourth quadrant and is calculated by the time probe takes to come out of sample and the force which is required to take the probe out of the sample. Table 4 presents TPA data for all the nine batches of NS oil cream. The force-time plots of formulation show that incorporation of NS oil in the cream enhances the aesthetic properties of cream along with its texture.

In vitro drug release studies

In vitro release study of batch F_3 was performed in phosphate buffer (pH 6.8) at 37±1°C. A cumulative percentage release of 60.39 % thymoquinone in 24 h was observed (Figure 6).

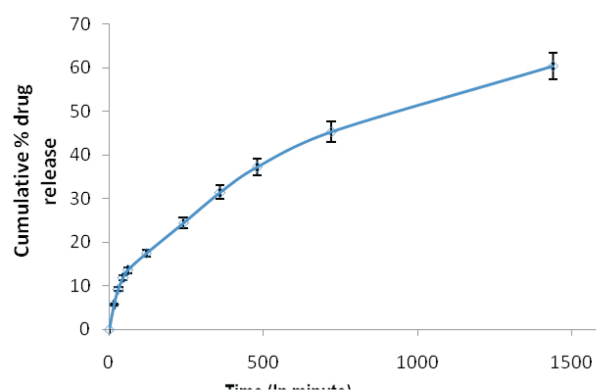

Figure 6: Cumulative % drug release profile of batch F_3 in phosphate buffer (pH 6.8) at 37±1°C over a period of 1440 minutes (24 h). (Mean ± SD, n=3)

The *in vitro* kinetic modeling of drug release data showed Higuchi release kinetics with a regression coefficient (R^2) of 0.996 indicating best linearity amongst various models applied (Table 5).

Table 5: Kinetic modeling of drug release data of thymoquinone from batch F_3 in phosphate buffer (pH 6.8) at 37±1°C

Zero Order Release	First Order Release	Higuchi Model	Korsmeyer Peppas Model
R^2	R^2	R^2	R^2
0.893	0.961	0.996	0.504

The value of release exponent (n) for Korsmeyer- Peppas model was found to be 0.504 indicating non-fickian diffusion type of release. The value of 'n' obtained by Korsmeyer- peppas model corresponds to 0.45<n<0.89 indicating non-Fickian transport [25].

In vitro occlusion test

The *in-vitro* occlusion test carried out at 32±1°C and 60±5% RH for a period of 12 h, indicated that emulsion base (in blank cream) exhibited moisture occlusive properties, but NS oil cream (batch F_3) prevented the loss of moisture better than blank cream formulation. The weight of moisture lost after 12 h of study have been listed in Table 6.

Table 6: *In vitro* occlusion test data for batch F_3 performed at 32 °C and 60±5% RH for 12 h

Groups	A (g)	B(g)	Occlusion Factor F	% water loss
Control	6.57	6.57	0	100
Black Cream*	6.60	4.30	34.84	65.15
F3	6.59	3.29	50.08	49.92

where, A= Solvent loss without sample (in grams) and B= Solvent loss with sample (in grams); n=3; S.D and C.V not presented in table; *p<0.05 (blank cream vs control); #p<0.05(NS oil cream vs control); and ^p<0.05(NS oil cream vs blank)

Significant difference (p<0.05) between the occlusive properties of blank and NS oil cream was observed; also, with respect to the control, the occlusive properties of blank and NS oil cream were significantly different.

Stability studies

Stability studies of optimal batch (F_3) were conducted at room temperature and 40 ± 2 ºC/ 75±5%RH for 90 days. The formulations were observed on 0th, 5th, 10th, 15th and 20th day for appearance, pH, consistency, removal and type of smear produced by them. There were no significant changes observed in the pH, color, appearance of the cream. The cream was found to be non-greasy and easily removed after application. No significant differences were observed between the stability batches and freshly prepared batches (of F_3).

Skin irritation studies

Cream of *Nigella sativa* oil (batch F_3) and blank cream, were applied to dorsal ear skin surface of rats and assessment was made on a scale of 0 to 14. No erythematic symptoms on the skin were observed upon application of cream, in addition, no significant differences were observed between untreated (control) and treated skin surfaces (Figure 7).

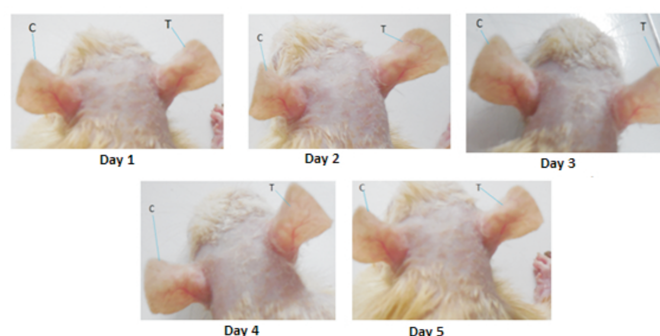

Figure 7: Skin irritation studies: Images of dorsal ear skin surface of female albino rats treated with NS oil cream (batch F_3). In figure, 'C' represents control (with no cream application) and 'T' represents treated skin surface. No significant difference (p > 0.05) was observed between control (C) and treated (T) skin surfaces.

Mean irritation score of NS oil cream was 3.0 ± 0.38 and that of blank cream was 3.1 ± 0.75.

Evaluation of dermatitis and skin lesions

NS oil cream treated skin lesions were imaged in order to evaluate its effect on CDNB-induced atopic dermatitis symptoms (Figure 8).

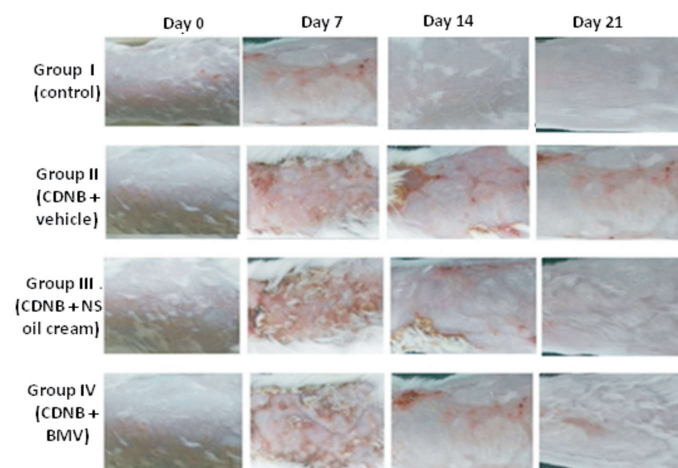

Figure 8: Images of AD skin lesions of treated groups of rats (1) Group I- naive control (vehicle), (2) Group II- CDNB +vehicle, (3) Group III- CDNB+NS oil cream (100 mg) and (4) Group IV- CDNB+ BMV (100 mg), on Day 0, 7, 14 and 21 of experiment

On the last day of experiment (day 21) dorsal skin surface of Group II showed presence of severe erythema, erosion, and dryness. However, groups III and IV showed smooth skin surfaces similar to that of Group I and both the groups (III and IV) showed comparable reduction in dermatitis symptoms.

CDNB induced considerable infiltration of inflammatory cells into epidermis and dermis. Hematoxylin and eosin stained sections of Group III (NS oil cream) showed marked reduction in thickening of skin and extent of inflammatory infiltrate in the dermis (Figure 9 c). The reduction noted was similar to that seen with the application betamethasone valerate cream in Group IV (Figure 9 d).

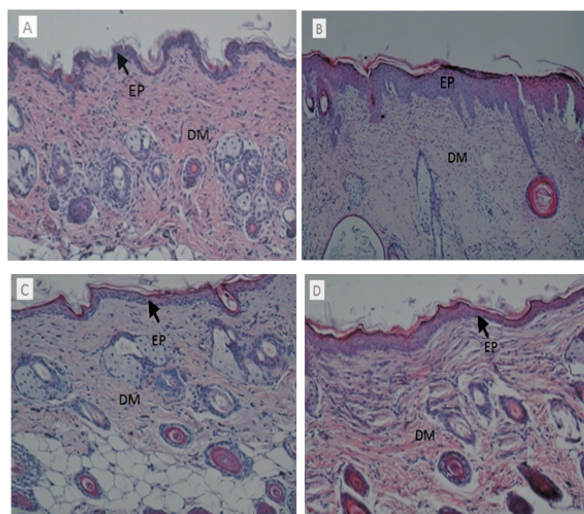

Figure 9: Histological images of AD-like skin lesions stained with hematoxylin and eosin. Sections were evaluated using microscope at an original magnification of 100X. For,(A) naive control (olive oil was used as vehicle), (B) CDNB+ vehicle, (C) CDNB+ NS oil cream, (D) CDNB+ BMV cream (0.1% w/w), observed thickness of epidermis (in mm) respectively were (A) 0.57 ± 0.037, (B) 2.10 ± 0.046, (C) 0.70± 0.038, and (D) 0.71± 0.034. Data represents mean ± SD (n=6). $p< 0.05$ (group II vs. group III); $p < 0.05$ (group II vs. group IV); and $p > 0.05$ (group III vs. group IV). EP represents epidermis; DM represents dermis.

Epidermal thickening induced as a result of dermatitis was also evaluated (Table 7).

Table 7: Evaluation of epidermal thickening induced by CDNB in different experimental groups

	Group I (naive control)	**Group II** (CDNB+ vehicle)	**Group III** (NS oil cream)	**Group IV** (BMV cream)
Thickness (in μm)	57.00± 3.67	210.00± 4.55	70.00± 3.58	71.00± 3.46

Data represents mean ± S.E.; n = 9

Maximum thickening was observed in Group II (210.00±4.55 μm). NS oil cream (Group III) abrogated thickening induced by CDNB. Significant ($p<0.05$) reduction in thickness of epidermis was found in Group III (70.00±3.58 μm) and IV (71.00±3.46 μm), in comparison to Group I (naive control) (57.00±3.67μm). Comparable reduction in thickness ($p > 0.05$) was observed in groups III and IV respectively.

Conclusion

Atopic dermatitis characterized by relapsing eczematous lesions and pruritus, shows typical infiltration of inflammatory cells into the epidermis and dermis which can be evaluated by staining with hematoxylin and eosin. Topical application of *Nigella sativa* oil cream induces no irritation and erythema; and also, demonstrated a significant alleviation in severity of AD-like symptoms in rats. Besides restoration of epidermal thickness, a decrease in infiltration of inflammatory cells into AD-like skin lesions was observed. The results of our studies suggest the efficacy of *Nigella sativa* oil cream in the treatment of atopic dermatitis.

Steroids have been used conventionally as a first line therapy for the treatment of AD; however, many side effects, such as atrophy, striae, rosacea, perioral dermatitis, acne and purpura, hypertrichosis, pigment alteration, delayed wound healing and exacerbation of skin infections, etc. are associated with long term treatment steroid therapy [26]. Even application of large amounts of topical steroids, does not result in mitigation of eczematic symptoms on skin [27]. A comparative study on treatment of hand eczema with *Nigella*, Betamethasone and Eucerin, showed comparable improvement in the symptoms with *Nigella* and Betamethsone. The study indicated that *Nigella* might have the same efficacy as Betamethasone in improvement of life quality and decreasing severity of hand eczema [28]. The efficacy of oral and topical *Nigella sativa* when studied in psoriasis patients, showed that the combination of ointment and oral doses of *Nigella sativa* achieved complete cure of lesions, excellent and good responses in 85% of patients, with a relapse rate of 18%. The study showed that *Nigella sativa* was well tolerated, with no side effects [29]. The fixed oil component of *Nigella sativa* has been effective in skin eruptions. Thymoquinone and fixed oil of *Nigella* has been found to inhibit eicosanoid generation and membrane lipid peroxidation [30]. The activity of *Nigella sativa* against photodermatoses showed efficient protection against skin reddening and burning [31]. Use of herbal therapy for treatment of skin disorders can definitely be considered as a safer alternative. The findings of our study clearly show that topical application of NS oil alleviates AD-like skin symptoms.

The observations made in our present study indicate that *Nigella sativa* oil cream mitigates AD-like skin lesions. Based on our findings we propose the use of NS oil cream for topical application in dermatitis skin conditions. The healing property of this cream may be attributed to inhibition of generation of inflammatory mediators by thymoquinone present in *Nigella sativa* oil.

Conflict of interest

The authors declare no conflict of interest.

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