METHODOLOGY

Materials

All the chemicals and materials used in the present investigation were either AR/LR grade or the best grade available in the laboratory and supplied by the manufacturers with out further purification or investigation. Details are given below.

Table 7: List of materials used in formulations

Sr.No.	Name of materials	Name of suppliers
1.	Vidang fruit	Sanjivani Aushdhalay, Bhavnagar, India.
2.	Embelin	Yucca Enterprise Ltd., Mumbai, India.
3.	Carbopol 934	S. D. Fine Chemicals Ltd., Mumbai, India.
4.	Propylene glycol	Finar Chemicals Pvt. Ltd., Ahmedabad, India
5.	Methyl paraben	Chemdyes Corporation, Rajkot, India.
6.	Sodium hydroxide	Chemdyes Corporation, Rajkot, India.
7.	Ethanol	Chemdyes Corporation, Rajkot, India.
8.	Triehanolamine	ACS Chemicals, Ahmedabad, India.
9.	White petrolatum	S. D. Fine Chemicals Ltd., Mumbai, India.
10.	White bees wax	S. D. Fine Chemicals Ltd., Mumbai, India.
11.	Stearic acid	S. D. Fine Chemicals Ltd., Mumbai, India.
12.	Propyl paraben	Chemdyes Corporation, Rajkot, India.
13.	Tween 80	Finar Chemicals Ltd., Ahmedabad, India
14.	Skin of albino guinea pig	Slaughter House, Bhuj, India

Table 8: List of chemicals used

Sr.No.	Name of chemicals	Name of suppliers
1.	Ammonia	Chemdyes Corporation, Rajkot, India.
2.	Lead acetate	Chemdyes Corporation, Rajkot, India.
3.	n-Hexane	Finar Chemicals Ltd., Ahmedabad, India
4.	n-Propenol	Finar Chemicals Ltd., Ahmedabad, India
5.	n-Butanol	Finar Chemicals Ltd., Ahmedabad, India
6.	Chloroform	ACS Chemicals, Ahmedabad, India.
7.	Potassium dihydrogen phosphate	Chemdyes Corporation, Rajkot, India.
8.	Disodium hydrogen phosphate	Chemdyes Corporation, Rajkot, India.
9.	Sodium chloride	Chemdyes Corporation, Rajkot, India.
10.	Dimethyl Sulfoxide	ACS Chemicals, Ahmedabad, India.
11.	Ferric chloride	Chemdyes Corporation, Rajkot, India.
12.	Agar	Chemdyes Corporation, Rajkot, India.
13.	Peptone	Chemdyes Corporation, Rajkot, India.
14.	Dextrose	Chemdyes Corporation, Rajkot, India.

Table 9: List of micro-organisms (cultures) used

Sr.No.	Name of micro-organisms	Name of suppliers
1.	*Candida Albicans*	MTCC, Inst. of microbial technology, Chandigarh.
2.	*Trychophyton Rubrum*	MTCC, Inst. of microbial technology, Chandigarh.
3.	*Microsporum Canis*	MTCC, Inst. of microbial technology, Chandigarh.

Equipments and Instruments

Table 10: List of equipments and instruments

Sr.No.	Name	Name of manufacturers
1.	Soxhlet apparatus	EIE, Ahmedabad, India.
2.	Digital pH meter	Welltronix Instruments, Ahmedabad.
3.	HPTLC	Model- CD-60, Desaga, Mumbai, India.
4.	UV-Visible spectrophotometer	Model- Helios α, Thermo Electronics, Bombay.
5.	Brookfield digital viscometer	Bombay Pharma Instruments, Mumbai, India.
6.	Magnetic stirrer	EIE, Ahmedabad, India.
7.	Digital weighing balance	Electrolab, Bombay, India
8.	Hot air oven	EIE, Ahmedabad, India.
9.	Mechanical stirrer	Bio-lab Instruments Mfg. Co. Mumbai.

4.1 Method for evaluation of Vidang fruit

4.1.1 Collection and authentication of Vidang fruit

The fruits of *Embelia Ribes* (Vidang) were purchased from Sanjivani Aushdhalay, Bhavnagar. Herbarium of Vidang fruit was prepared and authenticated by National Institute of Science Communication and Information Resources, New Delhi (NISCAIR). Finally, Herbarium was preserved in Department of Pharmaceutical Sciences, Saurashtra University, Rajkot.

4.1.2 Description of Vidang fruit powder[42, 118]

The powder of Vidang fruit was tested for general appearance like color, odour & taste. The preliminary evaluation for foreign matters was done to remove admixed material if present in sample as an adulterant or substitute by visualization method.

4.1.3 Quantitative standard[119, 120]

To ensure the good quality of Vidang fruit powder, various physicochemical parameters were evaluated.

Loss on drying

Weigh a glass-stoppered, shallow weighing bottle that has been dried under the same conditions to be employed in the determination. Transfer specifies quantity of powder sample to the bottle and weighs the bottle and the contents. Remove the stopper and place the loaded bottle in the drying chamber. Dry the powder for specified time and temperature. After drying, close the bottle and allow it to cool to room temperature. Weigh the bottle and contents.

Alcohol soluble extractive

Macerate 5 gm of the air-dried drug, coarsely powdered, with 100 ml alcohol of the specified strength in a closed flask for 24 hrs, shaking frequently during the first 6 hrs

and allowing to stand for 18 hrs. Thereafter, filter it rapidly with precautions against loss of alcohol, evaporate 25 ml of the filtrate to dryness in tared flat bottomed shallow dish, dry at 105^0C and weigh. Calculate the percentage of alcohol soluble extractive with reference to the air dried drug.

Water soluble extractive

Add 5 gm of powdered drug to 50 ml of water at 80^0C in a stoppered flask. Shake well and allow to stand for 10 min, cool, add 2 gm of kieselguhr and filter it. Transfer 5 ml of the filtrate to a tared evaporating dish, evaporate the solvent on a water bath, continue drying for 30 min, finally dry in a steam oven for 2 hrs and weigh the residue. Calculate the percentage of water soluble extractive with reference to the air dried drug.

Total ash

Weigh accurately 2 gm of the air dried crude drug in a tared silica dish and incinerate at a temperature not exceeding 450^0C until it become free from carbon, cool and weigh. If a carbon-free ash cannot be obtained in this way, exhaust the charred mass with hot water, collect the residue on an ashless filter paper, incinerate the residue and filter paper until the ash is white or nearly so, add the filtrate, evaporate to dryness and ignite at a temperature not exceeding 450^0C. Calculate the percentage of ash with reference to air dried drug.

$$\text{Total ash (\%)} = \frac{\text{Weight of ash}}{\text{Weight of drug taken}} \times 100 \qquad \text{(Equation 6)}$$

Acid insoluble ash

Boil the ash obtained from the procedure mentioned in the total ash with 25 ml of 2 M hydrochloric acid for 5 min, collect the insoluble matter in a Gooch crucible or on an

ashless filter paper, wash with hot water, ignite, cool in a desiccators and weigh. Calculate the percentage of acid insoluble ash with reference to air-dried drug.

$$\text{Acid insoluble ash (\%)} = \frac{\text{Weight of acid insoluble residue}}{\text{Weight of drug taken}} \times 100 \quad \text{(Equation 7)}$$

Table 11: Limits for quality parameters

Sr.No.	Parameters	Reference value
1	Loss on drying	Not more than 5.0 %
2	Alcohol soluble extractive	Not less than 3.0%
3	Water soluble extractive	Not less than 7.0%
4	Total ash	Not more than 5.0%
5	Acid insoluble ash	Not more than 1.0%

4.1.4 Chemical tests[42, 121]

Specific identification test (color reaction)

Take 0.5 gm powdered drug add 25 ml of diethyl ether. Shake and allow to stand for 2 hrs. Decant the supernatant ether layer. Add 1 ml of ammonia solution to the ethereal layer.

Lead acetate test:

Take the little quantity of the test solution and mix with basic lead acetate solution. Formation of the white precipitates indicates the presence of tannins.

Ferric chloride test:

To 1ml of the extract, add 5% ferric chloride solution, formation of a dark blue or greenish black color product which shows the presence of tannins.

4.2 Estimation of embelin by U.V. Spectrophotometrically

4.2.1 Determination of absorption maxima

A spectrum of the embelin was obtained by scanning the solutions of embelin in phosphate buffer (pH 7.4) containing ethanol from 270-370 nm against same reagent as a blank to fix absorption maxima.

4.2.2 Plotting of calibration curve

10 mg of embelin was accurately weighed and transfer to 100 ml volumetric flask and dissolved in phosphate buffer (pH 7.4) containing ethanol and volume of the flask was made up to 100ml with same solvent to get concentration of 100µg/ml.

The above stock solution (100 µg/ml) was further diluted to get concentration in the range of 20-100 µg/ml in phosphate buffer (pH 7.4) containing ethanol.

Absorbance of each solution was measured using UV-Visible double beam spectrophotometer by putting phosphate buffer (pH 7.4) containing ethanol as a reference standard. The calibration curve was generated for entire range of concentrations. The experiment was preformed in triplicate and based on average absorbance; the equation for the base line was generated.

4.3 Method for extraction of Vidang fruit[42]

Vidang extract was prepared by continuous extraction technique using Soxhlet's apparatus. 500 gm of Vidang powder was extracted with n-hexane at 40^0C and lastly remaining concentrated extract was collected and dried by using steam bath. Weighed and percentage yield of dry extract was determined.

4.4 Methods for evaluation of Vidang extract

4.4.1 TLC study of Vidang extract[42]

Assay was carried out to determine presence of embelin in Vidang extract in following chromatographic conditions as per Indian herbal pharmacopoeia.

Chromatographic condition:

Stationary phase **:** Aluminum-backed silica gel 60 F254 plates (E. Merck)

Mobile phase **:** n-Propenol: n-Butanol: 4N Ammonia (7: 1: 2)

Chamber saturation **:** 30 min

Reference solution **:** Dissolve 1 mg of embelin in chloroform.

Test sample **:** Dissolve Vidang extract in chloroform.

Visualization of spots **:** UV 254 nm

Sample ID **:** (a) Embelin

(b) Vidang extract

4.4.2 Assay of Vidang extract by HPTLC method[42]

Assay was carried out to determine presence of embelin and its content in extract of Vidang fruit in following chromatographic conditions as per Indian herbal pharmacopoeia. Finally, Concentration of embelin in Vidang extract was determined using the calibration curve plotted between concentration and peak area of standard embelin**.**

Chromatographic condition:

Instrument **:** HPTLC instrument with DESAGA Applicator, Densitometer

Stationary phase **:** Silica gel 60 F_{254} TLC plates

Mobile phase **:** n-propanol: n-butanol: 4N ammonia (7:1:2)

Chamber saturation time **:** 20 min

Spotting dimension **:** 5 mm

Distance between two spots **:** 10 mm

Wavelength **:** 333 nm

Syringe **:** 100 µl

Plate **:** 100 mm

Positions **:** 6

Start **:** 12 mm

Volume **:** 5 µl

Time **:** 10 s/µl

- **Standard preparation:**

Weigh 10 mg of embelin in 10 ml volumetric flask, dissolve in chloroform and make up volume with chloroform. Take 2ml of above solution in 10 ml volumetric flask and dilute to 10ml with chloroform. (200 µg/ml)

- **Sample preparation:**

Weigh 2 mg Vidang extract and transfer in 100 ml volumetric flask containing chloroform and finally dissolve and make up volume to 100 ml with chloroform.

4.5 Preparation, pharmatechnical evaluation and optimization of topical gel containing Vidang extract

4.5.1 Full factorial design[122]

A 3^2 randomized full factorial design was used in this study. In this design 2 factors were evaluated, each at 3 levels, and experimental trials were performed at all 9 possible combinations. The amount of carbopol 934 (X_1) and amount of propylene glycol (X_2) were selected as independent variables. A statistical model incorporating interactive and polynomial terms was utilized to evaluate the response.

$$Y = b_0 + b_1X_1+b_2X_2 + b_{12}X_1X_2 + b_{11}X_1^2 + b_{22}X_2^2 \quad \text{(Equation 8)}$$

Where, Y is the dependent variables, b_0 is the arithmetic mean response of the nine runs, and b_1 is the estimated coefficient for the factor X_1. The main effects (X_1 and X_2) represent the average result of changing one factor at a time from its low to high value. The interaction terms (X_1X_2) show how the response changes when two factors are simultaneously changed. The polynomial terms (X_1^2 and X_2^2) are included to investigate non-linearity.

4.5.2 Optimization of Vidang topical gel formulations using 3^2 full factorial design[85]

It is desirable to develop an acceptable pharmaceutical formulation in shortest possible time using minimum number of man hours and raw materials. Traditionally pharmaceutical formulations are developed by changing one variable at a time approach. The method is time consuming in nature and requires a lot of imaginative efforts. Moreover, it may be difficult to evolve an ideal formulation using this classical technique since the joint effects of independent variables are not considered. It is therefore very essential to understand the complexity of pharmaceutical formulations by using established statistical tools such as factorial design.

In addition to the art of formulation, the technique of factorial design is an effective method of indicating the relative significance of a number of variables and their interactions. The number of experiments required for these studies is dependent on the number of independent variables selected. The response/s (Yi) is/are measured for each trial and then either

Simple linear: ($Y= b_0+ b_1X_1 + b_2X_2+ b_3X_3$) (Equation 9)

or

Interactive: ($Y= b_0+ b_1X_1 + b_2X_2+ b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3$---) (Equation 10)

or

Quadratic: ($Y = b_0+ b_1X_1 + b_2X_2+ b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3$---$+ b_{11}X_1^2$---) (Equation 11)

Model is fitted by carrying out multiple regression analysis and F-statistics to identify statistically significant terms.

The full equation, an equation containing only statistically significant terms, is then used for drawing counter plots to visualize the impact of changing variables at a glance. The optimum point may be identified from the plot and replicate trials may be run to verify the prediction of optimum response. For simplicity, it was decided to perform a three variable study at three experimental levels to achieve the set objectives efficiently.

A 3^2 randomized full factorial design was utilized in the present study. In this design two factors were evaluated, each at three levels, and experimental trials were carried out at all 9 possible combinations. The coded values of independent factors and full factorial design layout are shown in table 12 and table 13 respectively. The amount of carbopol 934 (X_1) and amount of propylene glycol (X_2) were selected as independent variables. The chosen depended variables were viscosity, time required for 50% release of drug ($T_{50\%}$) and drug release at 1 hr (DR_1). The statistical analysis of factorial design batches

was performed by multiple linear regression analysis using Microsoft excel 2003. The formulations of the factorial batches (F1 to F9) are shown in table 13.

4.5.3 Preparation of Vidang topical gel

As per 3^2 factorial design (table 12), carbopol 934 as a gelling agents and propylene glycol as a co-solvent were used in varying proportion for the preparation of the gel. First, the required amount of carbopol 934 was added in to about 70 ml of water with vigorous stirring and left overnight for proper dissolving of the polymer and methyl paraben as a preservative was dissolved slowly into it. Secondly, Vidang extract was taken in the beaker containing ethanol and mentioned amount of propylene glycol and dissolved completely by using magnetic stirrer. The open end of the beaker was covered with aluminum foil to minimize the evaporation of volatile solvent. The carbopol 934 solution was slowly dispersed in the mixture of Vidang extract and co-solvent with vigorous stirring at 200 RPM. Again the beaker was covered with aluminum foil and stirred continue for 15 min. After that smooth dispersion was allowed to stand for removal of entrapped air. The gels were spontaneously formed with dropwise addition of triethanolamine with continuous stirring to avoid air entrapment. Finally, pH is adjusted using 1% w/v solution of sodium hydroxide. Then remaining quantity of water is incorporated in it and stored at ambient temperature prior to its use.

Table 12: Independent variables and their coded values

Independent variables	Coded values		
	-1	**0**	**1**
Amount of carbopol 934 polymer (gm) X_1	1.2	1.6	2.0
Amount of propylene glycol (gm) X_2	8	12	16

Table 13: Full factorial design layout

Formulation code	X_1	X_2
F1	-1	-1
F2	-1	0
F3	-1	+1
F4	0	-1
F5	0	0
F6	0	+1
F7	+1	-1
F8	+1	0
F9	+1	+1

Table 14: Vidang gel formulations using 3^2 full factorial design

Ingredients	Formulation code								
	F1	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**
Vidang extract (gm)	20	20	20	20	20	20	20	20	20
Carbopol 934 (gm)	1.2	1.2	1.2	1.6	1.6	1.6	2.0	2.0	2.0
Propylene glycol (gm)	8	12	16	8	12	16	8	12	16
Ethanol (ml)	2	2	2	2	2	2	2	2	2
Methyl paraben (gm)	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14
Triethanolamine (ml)	2	2	2	2	2	2	2	2	2
Sodium hydroxide (ml)	Q.S	Q.S	Q.S	Q.S	Q.S	Q.S	Q.S	Q.S	Q.S
Water up to (Q.S) (ml)	100	100	100	100	100	100	100	100	100

4.5.4 Evaluation of Vidang topical gel formulations[85, 123, 124]

Physical appearance

All topical formulations were visually checked for their color, consistency, homogeneity and phase separation.

pH measurement

4 gm of topical formulation was accurately weighed and dispersed in 100 ml of purified water. The pH of all formulations was determined by using pre-calibrated digital pH meter.

Viscosity measurement

Brookfield viscometer was used to measure viscosity of topical formulation. The spindle was rotated at 10 RPM. Samples of the topical formulations were allowed to settle over 30 min at the assay temperature ($25^0 \pm 1^0$C) before the measurements were taken.

Spreadability

Two glass slides of standard dimensions were selected. The topical formulation whose spreadability had to be determined was placed over one of the slide. The second slide was placed over this slide in such a way that the formulation was sandwiched between them across a length of 6 cm along the slide. 1000 gm weight was placed upon the upper slide so that the formulation between the two slides was pressed uniformly to form a thin layer. The weight was removed and the excess of the formulation adhering to the slides was scrapped off. The lower slide was fixed on the board of the apparatus and one end of the upper slide was tied to a string to which 20 gm load could be applied with the help of a simple pulley. The time taken for the upper slide to travel the distance of 6 cm and separate away from lower slide under the direction of the weight was noted. Spreadability was determined by equation:

$$\text{Spreadibility} = \frac{m \times l}{t} \quad \text{(Equation 12)}$$

Where, m = Weight tied to the upper slide (20gm)

l = Length of glass slide (6cm)

t = Time taken in seconds.

Drug content

Weighed approximately 1 gm of topical formulation and dissolved in about 40 ml of phosphate buffer (pH 7.4) in 100 ml volumetric flask with vigorously stirring and finally make the 100 ml volume with same solvent. From this stock solution, an aliquot of 1ml solution was accurately transferred into 10 ml volumetric flask and made up to volume with same solvent. The solution was filtered through a filter prior to UV analysis at 327 nm. Absorbance of resultant solution was measured using UV-Visible double beam spectrophotometer against phosphate buffer (pH 7.4) as a blank. Estimate the drug content in all topical formulations by using calibration curve.

***In vitro* diffusion study**

The release rate of the drug from topical formulation was determined using Franz diffusion cell. In Franz diffusion cell, 1 gm of topical formulation was kept in donor compartment. The entire surface of cellophane membrane was in contact with the receptor compartment containing 22ml of phosphate buffer (pH 7.4). The receptor compartment was continuously stirred using a magnetic stirrer at 100 RPM. The temperature maintained was $37^0 \pm 1^0$C. A sample (3 ml) of the solution was withdrawn from the diffusion apparatus at an interval of every 1 hr up to 8 hrs and same volume was replaced with fresh phosphate buffer. The samples were filtered through a 0.45 µ membrane filter. Absorbance of withdrawn samples was measured at 327 nm using UV-

Visible double beam spectrophotometer. Cumulative percentage of drug release was calculated by using calibration curve.

***Ex vivo* permeation study**

Franz diffusion cell mounted with skin of albino guinea pig was used for drug permeation study. 1 gm of optimized topical formulation was taken into the cell (donor compartment) and phosphate buffer (pH 7.4) in receptor compartment which is agitated using magnetic stirrer at 100 RPM and temperature maintained was $37^0 \pm 1^0$C. A sample (3 ml) of the solution was withdrawn from the diffusion apparatus at an interval of every 1 hr up to 8 hrs and same volume was replaced with fresh phosphate buffer. The samples were filtered through a 0.45 μ membrane filter. Absorbance of withdrawn samples was measured at 327 nm using UV-Visible double beam spectrophotometer. Cumulative percentage of drug release was calculated by using calibration curve.

***In vitro* antifungal study using cup-plate method**

In vitro antifungal studies of optimized topical formulation against *Candida Albicans* (MTCC-227), *Trychophyton Rubrum* (MTCC-296) *and Microsporum Canis* (MTCC-3270) were determined by agar diffusion method employing ' Cup Plate technique'. The cup plate method depends upon diffusion of antifungal formulations through a solidified agar layer in a petridish or plate to an extent such that growth of added micro-organism is prevented entirely in a zone around the cup containing anti fungal drug. A solution of optimized formulation in DMSO was poured into cups bored into sabouraud's glucose agar previously seeded with test organisms. After diffusion, the agar plates containing *Candida Albicans, Trychophyton Rubrum and Microsporum Canis* were incubated at 25^oC for 2 days, 30^oC for 7 days and 25^oC for 5 days respectively. The zone of inhibition

was observed for all plates and compared with control containing DMSO solvent only. The entire operation was carried out in aseptic condition throughout the study.

4.5.5. Stability studies[125, 126]

Stability studies of the optimized gel formulation were conducted for 3 months. The optimized formulation was filled in clear wide mouthed glass bottle and exposed to 25^{0}C/65% RH and 40^{0}C/75% RH in humidity control oven as per ICH guidelines. After specified time interval, the optimized formulation was evaluated for their pH measurement, viscosity measurement, drug content and *in vitro* diffusion study.

4.6 Preparation, pharmatechnical evaluation and optimization of topical cream containing Vidang extract

4.6.1 Optimization of Vidang topical cream formulations using 3^2 full factorial design[103]

A 3^2 randomized full factorial design was utilized in the present study. In this design two factors were evaluated, each at three levels, and experimental trials were carried out at all 9 possible combinations. The coded values of independent factors and full factorial design layout are shown in table 15 and table 16 respectively. The amount of stearic acid (X_1) and amount of propylene glycol (X_2) were selected as independent variables. The chosen depended variables were viscosity, time required for 50% release of drug ($T_{50\%}$) and drug release at 1 hr (DR_1). The statistical analysis of factorial design batches was performed by multiple linear regression analysis using Microsoft excel 2003. The formulations of the factorial batches (C1 to C9) are shown in table 16.

4.6.2 Preparation of Vidang topical cream

As per 3^2 factorial design (table 15), stearic acid and propylene glycol were used in varying proportion for the preparation of the cream. Preparation of cream was done by emulsifying procedure in which oil phase and aqueous phase was thoroughly mixed at the same temperature. In the separate vessel, oil phase and aqueous phase were prepared. In the oil phase, white petrolatum, white bees wax, stearic acid and tween 80 were weighed and melted in a porcelain dish at 65-70^{0}C. In the aqueous phase, water and propylene glycol were mixed and finally warmed at 65-70^{0}C. Add aqueous phase slowly drop by drop in oil phase and mixed properly on the magnetic stirrer at 600 RPM. Vidang extract was levigated in required quantity of propylene glycol and finally add into the mixture of both phase with slowly stirring for 10 min to achieve better consistency of cream formulation.

Table 15: Independent variables and their coded values

Independent variables	Coded values		
	-1	**0**	**1**
Amount of stearic acid (gm) X_1	2	6	10
Amount of propylene glycol (gm) X_2	2	7	12

Table 16: Full factorial design layout

Formulation code	X_1	X_2
C1	-1	-1
C2	-1	0
C3	-1	+1
C4	0	-1
C5	0	0
C6	0	+1
C7	+1	-1
C8	+1	0
C9	+1	+1

Table 17: Vidang cream formulations using 3^2 full factorial design

Ingredients	Quantity								
	C1	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**	**C8**	**C9**
Vidang extract (gm)	20	20	20	20	20	20	20	20	20
Oil phase									
White petrolatum (gm)	25	25	25	25	25	25	25	25	25
White bees wax (gm)	5	5	5	5	5	5	5	5	5
Stearic acid (gm)	2	2	2	6	6	6	10	10	10
Tween 80 (ml)	1	1	1	1	1	1	1	1	1
Aqueous phase									
Propylene glycol (gm)	2	7	12	2	7	12	2	7	12
Methyl paraben (gm)	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
Propyl paraben (gm)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Water up to (Q.S) (ml)	100	100	100	100	100	100	100	100	100

4.6.3 Evaluation of Vidang topical cream formulations

Vidang topical cream formulations were evaluated for physical appearance, pH measurements, viscosity measurements, spreadability and drug content as per method described in section 4.5.4.

***In vitro* diffusion study**

The release rate of the drug from Vidang topical cream formulations was determined using Franz diffusion cell. The method for *in vitro* diffusion study was followed as per section 4.5.4.

***Ex vivo* permeation study**

The drug permeation study of optimized cream formulation was carried out using Franz diffusion cell mounted with skin of albino guinea pig. The method was followed for *Ex vivo* permeation study as per section 4.5.4.

***In vitro* antifungal study using cup-plate method**

In vitro antifungal studies of optimized cream formulation against *Candida Albicans* (MTCC-227), *Trychophyton Rubrum* (MTCC-296) *and Microsporum Canis* (MTCC-3270) were determined by agar diffusion method employing ‘ Cup Plate technique’. The method for *in vitro* antifungal study was followed as per section 4.5.4.

4.6.4 Stability studies

Stability studies of the optimized cream formulation were conducted for 3 months as per method describe in section 4.5.5.