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Research Article

Formulation and Characterization of Herbal Nanogel for Fungal Skin Infections

Vipul Tiwari, Surendra Pratap Singh*, Jitendra Kumar Malik
Faculty of Pharmacy, P K University, Shivpuri (M.P.) 473665

ABSTRACT
The present study provides a novel herbal Nano formulation for the treatment of fungal skin infections. Extraction of the medicinal plants (Pipper longum, Phyllanthus niruri and Berberis aristata) and C. lanatus seed oil was carried out. After the extraction process, the extracts were used for quantitative and qualitative assessment of Phytoconstituents, their antioxidant and anti-inflammatory potential. Moreover, the GCM Sprofiling of the oil extracted from C. After that, plant extracts nanoemulsion formulation was prepared with different biopolymers namely sodium alginate, chitosan and gum arabic. On the basis of physical evaluation, stability parameters and low droplet size, gumarabic based nanoemulsion with varying concentration of oils was selected to formulate into nanogel with two gelling agents i.e xanthan gum and guar gum. Nine different ratios of xanthan gumand guar gumwere formulated with five different ratios of oils i.e (1%, 3%, 5% and 10%). After that on analyzing physical evaluation, droplet size and polydispersity index characterization. Out of total 72 ratios, 36 ratios of xanthan gum and 36 ratios of guar gumit was found that 2:1:1:1 ratio of guar gumnanogel and 1:1:1:1 ratio of xanthangum nanogel showed good stability, *in vitro* drug release studies showed that the *B. aristata* guar gum based herbal nanogel showed higher drug release rate which permitted a faster rate of drug dissolution into the aqueous phase. The release kinetics fitting data indicated that drug release followed the Quasi Fickian and Fickian diffusion release model. Further, *in vitro* antidermatophytic activity of optimized herbal nanogel against *T. rubrum* and *T.mentagrophytes* was carried out**.** The results revealed that guar gum nanogel formulated with *B. aristata* showed significant zone of inhibition as compared to *B. aristata* xanthan gum nanogel and ketoconazole against *T. rubrum*. *In vivo* studies had confirmed the prepared *B. aristata* nanogel retarded the growth of *T. rubrum*. In the end stability studies was carried out indicating that the *B. aristata* guar gum based nanogel formulation is stable and found to be consistent with no change in drug content, pH values, phase separation, and transparency for upto 3 months. Thus, our *B. aristata* nanogel is a safe, effective and promising formulation for the topical treatment of dermatophytic skin infections.

KEYWORDS: Antifungal, Herbal plants, Soxhlet apparatus, Abieswebbiana Pipper longum, Phyllanthus niruri and Berberis aristata

1. INTRODUCT

In today's age, fungal infection is a rising cause of concern as a large population is affected by it[1]. In the world, more than a billion people suffer from a number of fungal infections. More than 80 percent of the human population is at risk of fungal infections, according to the leading international fungal education (LIFE) portal[2]. The human body can be affected by two main types of fungal infections, i.e., topical and systemic. In tropical and sub-tropical countries where the climate is hot and humid, topical fungal infections are very prevalent and skin, hair, nails and scalp are mostly affected by the topical infection. Systemic fungal infections, the second type, are more lethal as it is the primary cause of the elevated rate of mortality and morbidity worldwide [3].

Several groups of researchers had studied the biological function of medicinal plants from all over the world. These studies were focused on the common uses of different species, as well as on popular knowledge and empirical studies explaining the use of medicinal plants, with an emphasis on how these plants could support the pharmaceutical industry [4]. Around 50 percent of the drugs licensed nowadays were derived directly or indirectly from natural products [5]. For the effectiveness of a formulation, the chemical complexity of extracts is an extremely important factor, since the active ingredient must also be

***Corresponding Author:**
Surendra Pratap Singh
Faculty of Pharmacy, P K University Shivpuri (M.P.) 473665
Email: spspharma2001@gmail.com
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released from the formulation. Therefore, potent vehicles or carriers must be used to simultaneously increase the drug's solubility, reduce the process of degradation, decrease any toxicity, and thus monitoring the successful absorption and biological reaction[6].

To overcome all these problems, medicinal plants can be taken into account as the potent and promising therapeutics for the improvement of fungal skin infection treatments based on the variety of the active and effective components such as flavonoids, essential oils, alkaloids, phenolic compounds, terpenoids, fatty acids[7,8].

Nanogels are gaining importance day by day as they have dramatic advantages for targeting any drug in a vast number of diseases. They posses both the hydrogel and Nanocarrier's properties. Nanogels have utilized not only for superficial acute diseases, but also entered the critical fatal disease therapies[9]. These delivery systems are now curing the brain disorders, lung and liver disorders, cancers, skin diseases, joint disorders, ophthalmic, wound healing and vaccine delivery. The present study has been designed for the formulation and characterization of herbal nanogel for the treatment of fungal skin infections [10].

Material and Method

Sample Collection

The *Citrullus lanatus* seed was purchased from a local market of MP (India). The plants *Phyllanthus niruri*, *Pipper longum* and *Berberis aristata* were collected from different places of Madhya Pradesh[11]. All the medicinal plants and herbs were identified and authenticated from the Botanical survey of India, Bhopal having Voucher specimen no. 06, 07 and 09. Plants and their parts used in the study[12].

Plant Extract Preparation

The parts of *Pipper longum* (fruits), *Phyllanthus niruri* (whole plant) and *Berberis aristata* (Bark) were utilized for preparation of extracts. Plants parts were surface sterilized with 0.1% $HgCl_2$ and then washed with triple distilled water for 8-10 minutes[13].

Plant leaves, seeds and roots were dried for 1 to 2 weeks at room temperature. Dried plant part was grind to make fine powder. The plants material was extracted with 100% polar solvents methanol (5.1) at 68°C by Soxhlet method. Solvents evaporation was done by rotary evaporator (Ingle *et al*., 2017). After evaporation the plant material was stored at 4°C in refrigerator for further use. The percentage yield of plant material was calculated by following equation[14]:

% Yield of the extract = $(Plant\ material\ weight\ after\ extraction\ process\ Plant\ material\ weight\ taken\ for\ extraction)\ X\ 100$

Test organism and inoculum preparation

The fungal pathogens Trichophyton rubrum (NCCPF no. 900001) and Trichophyton mentagrophytes (NCCPF no. 800009) was obtained from the National Culture Collection of Pathogenic Fungi (NCCPF), Chandigarh (India), and was maintained on Sabouraud dextrose agar (SDA) (Himedia). The slant medium was supplemented with 0.1 % yeast extract and stored at 4 ˚C for six months as active cultures and revived after that[11]. For inoculum preparation, stock suspensions of these fungal pathogens were prepared from a sporulating 15-days old culture grown on the SDA plate at 28-30 ℃. The colonies were enclosed with 5ml of sterile distilled water, having 0.05% Tween 20 and surface scraped with a sterilized loop. The conidia and hyphal fragments mixture was filtered with a sterile filter of 8 mm pore size and collected in a sterile test tube[12]. This method removed the most of the hyphae and gives rise to inocula composed mainly of spores. The suspension was allowed to stand for 10 min for the sedimentation of hyphal fragments, and from the supernatant, inoculums were prepared by adjusting to a 0.5 McFarland turbidity standard (1.0×106 spores ml-1) by using a haemocytometer.

***In vitro* antidermatophytic activity of *C. lanatus* seed oil and different plant extracts against dermatophytes**

The antifungal potential was evaluated by agar well diffusion method (Das and Jha, 2013). In brief, 25 ml of sterilized SDA media poured into the Petri plates. 10 µl of fungal suspension was uniformly spread over solidified SDA plates by using a sterilized spreader. The wells having a diameter of 6 mm were made in the center of these SDA plates by sterilized cork borer. The 100 µl of the C. lanatus oil and 100 mg of different plant extracts were dissolved in DMSO, was loaded into the wells and permitted to diffuse at room temperature for an hour[13]. Pure DMSO was used as a negative control, and for positive control, Ketoconazole (10 mg) was used. Then, for 3 days, the plates were incubated at 28- 30℃. Antifungal activity was evaluated around the well that was filled with the oil by measuring the diameter of the inhibition zone.

Determination of Minimum inhibitory concentration (MIC)

The MIC against fungal pathogens was done by the broth microdilution reference method after the evaluation of the antifungal activity of the oil by agar well diffusion method (CLSI 2002). The microdilution assay was carried out in SDA medium using approximately 1.0×106 spores ml-1. The 100 µl of SDB was loaded into every well of 96- well plate, and further, the first well was supplemented with 100 µl of oil and plant extracts[14]. Subsequently, two-fold dilutions were done using a micropipette, and then an inoculum volume of 10 µl was added to every well except for

negative control. The positive control of antibiotic (10 mg/ml), negative control (SDB), the broth alone, and the inoculum alone were also put in the experiment. The plate was sealed with parafilm, on a shaker agitated for 30 seconds to mix the content and after that incubated for three days at 28 - 30℃, plates were read visually. The lowest oil concentration where no color/turbidity change was observed indicates no growth of microorganisms and representing MIC and for quantitative analysis, OD was taken at 520 nm[15].

Optimization of *C. lanatus* oil for nanogel formulation with different biopolymers
Preparation of plant extract with sodium alginate based nanoemulsion formulation

Oil in water nanoemulsion was prepared by low energy method by with some modifications. For the preparation of plant extract and sodium alginate based nanoemulsion briefly 1% w/v of sodium alginate solution was prepared in distilled water to form homogeneous solution used as an aqueous phase. A fixed amount of plant extract i.e *Berberis aristata*, *Phyllanthus niruri* and *Pipper longum* were dissolved in different concentrations of *C. lanatus* seed oil (i.e 1%, 3%, 5% and 10%) and completely dissolved using a magnetic stirrer and was used as the dispersed oil phase. This oil phase was then mixed with aqueous phase (1% sodium alginate and 1% tween 80). The mixture was volume up to 25 ml by addition of double distilled water[16]. Then, the coarse mixture was subjected to magnetic stirrer at 1200 rpm upto 30 minutes. Along with this, a blank nanoemulsion with the same surfactant, oil and aqueous phase without extract was prepared following the same procedure, and it was considered as a control. The composition of sodium alginate and plant extract based nanoemulsion with different oil ratios was tabulated. Prepared samples were stored in glass vials at room temperature (25 ℃) for further analysis.

Preparation of plant extract with gum arabic based nanoemulsion formulation

For the preparation of plant extract and gum arabic based nanoemulsion briefly 1% w/v of gum arabic solution was prepared in distilled water and used as an aqueous phase. A fixed amount of (1%) plant extract i.e *Berberis aristata*, *Phyllanthus niruri* and *Pipper longum* were dissolved in different concentrations of *C. lanatus* seed oil (i.e 1%, 3%, 5% and 10%) and completely dissolved using a magnetic stirrer and was used as the dispersed oil phase. This oil phase was then mixed with aqueous phase (1% gum Arabic and 1% tween 80). The mixture was volume up to 25 ml by addition of double distilled water. Then, the coarse mixture was subjected to magnetic stirrer at 1200 rpm upto 30 minutes. Along with this, a blank nanoemulsion with the same surfactant, oil and aqueous phase without extract was prepared following the same procedure, and it was considered as a control. The composition of gum arabic and plant extract based nanoemulsion with different oil ratios was tabulated. Prepared samples were stored in glass vials at room temperature (25℃) for further analysis[17].

Preparation of plant extract with chitosan based nanoemulsion formulation

Oil in water nanoemulsion was prepared by low energy method by Liu *et al*., 2016 with some modifications. For the preparation of plant extract and chitosan based nanoemulsion briefly 1% w/v solution of chitosan was prepared using 1% acetic acid solution and used as a aqueous phase. A fixed amount of (1%) plant extract i.e *Berberis aristata*, *Phyllanthus niruri* and *Pipper longum* were dissolved in different concentrations of *C. lanatus* seed oil (i.e 1%, 3%, 5% and 10%) and completely dissolved using a magnetic stirrer and was used as the dispersed oil phase. This oil phase was then mixed with aqueous phase (1% chitosan and 1% tween 80). The mixture was volume up to 25 ml by addition of double distilled water[18]. Then, the coarse mixture was subjected to magnetic stirrer at 1200 rpm upto 30 minutes. Along with this, a blank nanoemulsion with the same surfactant, oil and aqueous phase without extract was prepared following the same procedure, and it was considered as a control. The composition of chitosan and plant extract based nanoemulsion with different oil ratios was tabulated. Prepared samples were stored in glass vials at room temperature (25 ℃) for further analysis[19]

Table1: Composition of chitosan, gum arabic, sodium alginate and plant extract based nanoemulsion with different oil ratios

S.No.	Chitosan (%)	Tween 80(%)	Oil (%)	Plant extract (%)	Total volume
1	1	0.5	1	1	25ml
2	1	0.5	3	1	25ml
3	1	0.5	5	1	25ml
4	1	0.5	10	1	25ml

S.No.	Gumarabic (%)	Tween80 (%)	Oil (%)	Plant extract(%)	Total volume
1	1	0.5	1	1	25ml
2	1	0.5	3	1	25ml
3	1	0.5	5	1	25ml
4	1	0.5	10	1	25ml

S.No.	Sodium alginate (%)	Tween80 (%)	Oil(%)	Plant extract(%)	Total volume
1	1	0.5	1	1	25ml
2	1	0.5	3	1	25ml
3	1	0.5	5	1	25ml
4	1	0.5	10	1	25ml

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Stability studies of prepared nanoemulsions

Stability studies of the prepared nanoemulsions were carried out by heating-cooling cycle, centrifugation and freeze-thaw cycle (Shah *et al*., 2010).

i. Heating-cooling cycle: Six cycles between refrigerator temperature 4°C and 45°C with storage at each temperature for 48 h were conducted and the formulated nanoemulsions were examined for stability (transparent with no phase separation).

ii. Centrifugation test: The formulated nanoemulsions were centrifuged at 3,500 rpm for 30 min observed for transparency and absence of phase separation.

iii. Freeze-thaw cycle: Three freeze-thaw cycles of the nanoemulsions between -21°C and +25°C for 48 h were performed and observed for transparency and absence of phase separation. Nanoemulsion formulations that passed the stability studies were selected for further characterization

Characterization of formulated nanoemulsion

Droplet size and polydispersity index (PDI)

The droplet size and polydispersity index (PDI) was determined using Zetasizer Nano ZS Particle Sizer (Malvern Instruments Ltd, Malvern, WR14 1XZ, UK) at a temperature of 25°C. The 1ml nanoemulsion was suitably diluted in triple distilled water before taking the droplet size and three measurements were acquired for each sample.

Formulation of Guar gum and Xanthan gum based nanogels

Nanogels were finally formulated from above prepared different concentration of oil based nanoemulsion formulations. In general, two different hydrophilic polymers like guar gum and xanthan gum were used to formulate the gel for topical application. Different concentrations of natural and semisynthetic polymers were postulated by adding pectin, carboxymethylcellulose (CMC) which was used as a gelling agent. Finally, the solution was plasticized with addition of glycerol. The composition of formulation was tabulated in Table 2. The gel stage is mixed into the nanoemulsion stage to procure nanogel. Incorporation of above prepared nanoemulsion into gel base with steady blending. The mixture was subjected to magnetic stirrer at 1200 rpm upto 30 minutes to form nanogel. Same procedure was used to formulate the xanthan gum based nanogels. The pH of prepared nanogel formulas was adjusted using triethanolamine (TEA) with stirring until desired pH value was approximately reached (6.1-6.5). Prepared samples were stored in glass vials at room temperature (25°C) for further analysis.

Table 2: The composition of nanogel formulation with xanthan gum and guar gum

S.NO.	Formulations (Ratios)	Ingredients			
		GuarGum/ Xanthan gum(ml)	Pectin (mg)	Glycerol (µl)	Carboxymethyll cellulose (CMC)(mg)
1	(1:1:1:1)	250	250	250	250
2	(2:1:1:1)	500	250	250	250
3	(2:2:1:1)	500	500	250	250
4	(2:1:2:1)	500	250	500	250
5	(3:1:1:1)	750	250	250	250
6	(3:2:1:1)	750	500	250	250
7	(3:3:1:1)	750	750	250	250
8	(3:1:2:1)	750	250	500	250
9	(3:1:3:1)	750	250	750	250

Physical evaluation and characterization of formulated Nanogel

Homogeneity

After placing the gels in the container, all formulations were tested for color, homogeneity (aggregates presence and appearance) by inspecting visually.

Droplet size and polydispersity index

The droplet size and PDI was determined using Zetasizer Nano ZS Particle Sizer (Malvern Instruments Ltd, Malvern, WR14 1XZ, UK) at a temperature of 25°C. The 1% nanogel was suitably diluted in triple distilled water before taking the droplet size and three measurements were acquired for each sample.

***In-vitro* drug release study of formulated nanogels**

In vitro drug release for the nanogel formulation was performed in phosphate buffer (pH 6.8) using dialysis membrane (Mali *et al*., 2017). The membrane (pore size: 12 KD, Sigma Chemical Co., USA) was activated by keeping it in phosphate buffer solution overnight. It was exposed to running water for few hours to remove glycerin based contents. Quantity of 5 g of all prepared hydrogel formulations were individually packed into dialysis tube with the ends being tightly

fastened and placed in a 150 ml of 10 mM phosphate buffer solution (pH 7.4) in a shaking incubator (maintained at 100 rpm and 37 ± 0.5°C). 1ml sample was withdrawn at predetermined intervals and replaced with same volume of the fresh buffer solution. The samples were then filtered and assayed for the drug content at 210 nm on UV/Visible spectrophotometer against the control. Absorbance was converted to drug concentration using a linear equation of calibration curve and then the cumulative percentage released was calculated taking into consideration the dilution factor. All measurements were performed in triplicate (n = 3).

Release kinetics study

To study the release kinetics of different nanogels formulations, the data obtained from the *in vitro* release study were analysed using various kinetic models to describe the mechanism of drug release from the hydrogels. Four kinetic models, (1) Zero-order, (2) Firstorder, (3) Higuchi square root models and (4) Korsmeyer-Peppas semi-empirical model, were applied on the release data as follows:

Zero order equation:

Qt = kot ..... (1)

Where Qt stands for the percentage of drug released at time t and ko is the release rate constant

II. First order equation:

Log Q=Log Q0-kt/2.303…….(2)

Where, Q0= is the initial concentration of drug, k= is the first order rate constant, t = release time;

III. Higuchi's equation:

Qt= kH t 1/2

Where kH represents the Higuchi release rate constant;

IV. Korsmeyer-Peppas semi empirical model was applied:

Qt/Qe=Ktn

Where Qt/Qe is the fractional drug release from the gel into the receptor media, K is a constant corresponding to the structural and geometric characteristics of the device and n is the release exponent which is indicative of the mechanism of the drug release. The (n) value of 0.5 that indicates Quasi-Fickian diffusion mechanism, while if (n>0.5) then anomalous or non-Fickian diffusion mechanism exists and if it is (=1) then the Zero order release one exists (Sabri *et al*., 2016).

***In vitro* antidermatophytic activity of optimized nanogel against dermatophytes**

The antifungal potential was evaluated by agar well diffusion method. In brief, 25 ml of sterilized SDA media poured into the Petri plates. 10 µl of fungal suspension was uniformly spread over solidified SDA plates by using a sterilized spreader. The wells having a diameter of 6 mm were made in the center of these SDA plates by sterilized cork borer. The 100 mg of formulated nanogel was loaded into the wells and permitted to diffuse at room temperature for an hour. Pure DMSO was used as a negative control, and for positive control, Ketoconazole marketed gel was used. Then, for 3 days, the plates were incubated at 28- 30°C. Antifungal activity was evaluated around the well that was filled with the oil by measuring the diameter of the inhibition zone.

***In vivo* evaluation**

Albino Wistar rats (Female) about 10-12 weeks old (150–250 g), were procured from Animal house facility, National Institute of Pharmaceutical Education and Research (NIPER) Mohali and were housed at Animal house facility, Shoolini University, Solan, H.P (India). The rats were acclimatized for a week prior to the initiation of the experiment in standard steel wire cages, fed with pellet diet (Ashirwad Industries, Chandigarh, India) and water was given *ad libitum.* The rats were kept under laboratory conditions temperature (25 ± 2ºC, relative humidity 45 ± 5 %, and a photoperiod of 12 h). The studies were conducted after obtaining ethical clearance from Institutional Animal Ethics Committee (IAEC), Shoolini University, Bajhol, Solan vide protocol approval no. IAEC/SU-19/19 and conducted according to Committee for the Purpose of Control and Supervision of Experiments on Animals CPCSEA (Govt. of India), New Delhi guidelines.

***In vivo* Skin irritation test**

Skin reversible damage subsequently by using of a gel sample known as skin irritation. Primary irritation is due to irritant which cause inflammation. These irritant involve the interaction of chemicals with sensory receptors in the skin at the site of application. Secondary irritation cause progressive inflammation on repeated contact. A variety of substances are used in the manufacture of topical gels. Therefore, finished products when used on the human body have the potential for several types of adverse reactions. The adverse effects that may be caused include skin irritation and allergic conditions, contact urticaria and photoallergy. Skin irritation potential for optimized nanogel was evaluated by carrying out skin irritancy test on Wistar albino rats (200–300 g). These rats were allowed to adopt the conditions for seven days before the commencement of the study. The study was carried out according to reported method by Pople and Singh, 2006. About 5 cm2 area on the dorsal surface of the rat was made hairless without damaging the skin surface, 4 h prior to the experiment. The shaved area was cleaned, and was treated with 100 mg of the optimized guar gum based *B. aristata* nanogel. The control group was left as untreated. Thus, the applied sites after 24, 48 and 72 h were observed for dermal reactions such as inflammation, erythema and edema. The mean erythemal and edemal scores were recorded on the basis of their degree of severity caused by application of

formulations: no erythema/edema = 0, slight erythema/ edema = 1, moderate erythema/edema = 2 and severe erythema/ edema =3.

***In vivo* anti dermatophytic evaluation**

Trichophyton rubrum was used as an infectious agent since it the most common fungus affecting number of population. The obtained fungal species was maintained on SDA at 4 ℃, and sub cultured once a month. For inoculum preparation, stock suspension of *T. rubrum* was prepared from a sporulating 15-days old culture grown on the SDA plate at 28-30 ℃. The colonies were enclosed with 5ml of sterile distilled water, having 0.05% Tween 20 and surface scraped with a sterilized loop. The conidia and hyphal fragments mixture was filtered with a sterile filter of 8 mm pore size and collected in a sterile test tube. This method removed the most of the hyphae and gives rise to inocula composed mainly of spores. The turbidity of the final inocula was made to 1.0×106 spores ml-1. An area of 5 cm2 was depilated on the dorsal surface of each animal. The 7 day old cultured infectious inoculum of *T. rubrum* was applied on to the depilated area and left for 6 days. The establishment of active infection was confirmed by visual examination such as scales, redness and erythematous lesions was observed in the animals on the 7th day.

The treatment was started on the 7th day post inoculation as the active infections were confirmed, and continued until complete recovery from the infection was achieved. All the animals were treated once daily with the formulated gel and marketed gel and the animals were kept undisturbed for 24hrs. The infected areas were scored visually for inflammation and scaling as well as for the presence of the pathogens, by day-to-day cultivation of skin scales from infected loci in SDA plates containing 100 units/ml of penicillin and streptomycin. The therapeutic efficacy of the ointment and gels were evaluated daily by macroscopic examination of lesions. Animals were divided into different groups of four animals each as shown in table 3

Table 3: Grouping of animals for antidermato phytic evaluation

S.No.	Group (n=4)	Treatment
1	Group 1 (control)	Vehicle control
2	Group 2 (Reference)	Conventional formulation of Ketoconazole (1%)
3	Group 3 (Tested group)	Optimized herbal nanogel formulation (1%)

Histology examination of skin specimens

The skin samples from treated and untreated (control) areas were taken. Each skin sample was stored in 10% (v/v) formalin saline solution. The skin samples were cut vertically in different sections. Each section of skin sample was dehydrated using ethanol and hematoxylin and eosin stain was used to study the morphological changes using phase contrast microscope fitted with a digital camera and compared with the control sample.

Stability studies

The optimized nanogel was stored at 4°C, 25℃ and 40°C for 3 months and samples were evaluated for physical parameters like turbidity, color change, pH and physicochemical parameters, drug content at one month interval. Any changes in evaluation parameters, if observed were noted.

Statistical analysis

All experiment was performed in triplicates with at least three to four concentration and results were expressed as Mean ± standard error mean (SEM). IC50 was calculated by excel and graph was plotted using prism 5.02. The data was analyzed by one-way ANOVA followed by Dunnett's test and P <0.05 considered as significant

RESULT AND DISCUSSION

Plant Extract Preparation

The yield of *C. lanatus* seed oil, *Pipper longum* (whole plant), *Phyllanthus niruri* (whole plant) and *Berberis aristata* (Bark) were found out to be 4.1%, 15.2%, 9.1% and 10.5 respectively (Table 4). The percentage yield of all the plants extract may depend on several factors which includes polarities of solvents, type of plant extract, solubility of plant and physical assets of the plants.

Table 4: Percentage yields of *C. lanatus* seed oil, *Pipper longum*, *Phyllanthus niruri* and *Berberis aristata*

S.No	Plant name	Part used	Extraction process	Final % yield
1	*C. lanatus* seed oil	Seeds	Water Distillation	4.1
2	*Pipper longum*	Fruit	Solvent distillation using methanol	15.2
3	*Phyllanthus niruri*	Whole Plant	Solvent distillation using methanol	9.1
4	*Berberis aristata*	Bark	Solvent distillation using methanol	10.5

Qualitative screening of Phytoconstituents

The phytochemical screening of the obtained plant extracts was qualitatively screened out for the presence of alkaloids, flavonoid, phenolics, amino acids, proteins, carbohydrates, saponins, glycosides, and phytosteroids. It was observed from qualitative analysis that the plant extracts consist of the most of the Phytoconstituents listed in the table 5.

Table 5: Screening of Phytoconstituents in the extract of *Pipper longum, Phyllanthus niruri* and *Berberis aristata*

S. No	Phyto-constituents	Qualitativeassay	*Pipper longum*	*Phyllanthus niruri*	*Berberis aristata*
1	Alkaloids	Wagner's test	+	+	+
2	Phenolic compounds	Ferricchloride	+	+	+
3	Glycosides	Salkowski Test	+	+	+
4	Carbohydrates	Molisch's Test	+	+	+
5	Proteins	Millon test	+	+	+
6	Amino acid	Ninhydrin test	+	+	+
7	Phyto steroids	Libermann-Burchard	-	+	+
		Salkowski reaction	-	+	+
8	Flavonoid test	Ferric Chloride Test	+	+	+
9	Saponins	Foam test	-	+	+
10	Tannin	Ferric Chloride Test	+	+	+
11	Fixed fats and oils		-	-	+

(+)* = Represents the presence of phytoconstituents and (-)* = Represents the absence of phytoconstituents

Table 6: Minimum inhibitory concentration of *C.lanatus* oil, *Pipper longum*, *Phyllanthus niruri* and *Berberis aristata* against dermatophytic strains

Minimum inhibitory concentration (MIC)		
Fungalpathogens	*Trichophytonmentagrophytes*	*Trichophytonrubrum*
P.longum (mg/ml)	25	12.5
P.niruri (mg/ml)	12.5	6.25
B.aristata (mg/ml)	6.25	1.56
*C.lanatus*oil (µl/ml)	12.5	12.5
Ketoconazole (µg/ml)	3.125	3.125

Preparation of plant extract with sodium alginate, gum arabic and chitosan based nanoemulsion formulation

The plant extracts *Pipper longum*, *Phyllanthus niruri* and *Berberis aristata* based nanoemulsions were successfully prepared by the low energy method. The nanoemulsion was prepared with different biopolymers i.e sodium alginate, gum arabic and chitosan in a ratio of 1% and non ionic surfactant tween 80 (0.5%). Different combinations ratios of all three biopolymers, *C. lanatus* oil and plant extract were. The blank nanoemulsion as a control was also prepared without plant extract. Nanoemulsion loaded with *P. niruri* extract showed light brown color, *P. longum* showed light greenish appearance and *B. aristata* loaded nanoemulsion showed light yellowish color while nanoemulsion prepared without plant extract i.e. control showed milky white color

Stability studies

The nanoemulsion formulations that were physically unstable showed the signs of phase separation and were rejected. The results of stability studies of different composition of nanoemulsion formulations prepared are shown in table 7,. The chitosan and sodium alginate based plant extract nanoemulsions with different oil ratios showed the signs of phase separation and was found to be unstable. Whereas, the gum arabic and plant extract based nanoemulsion with different oil ratios found to be stable with no phase separation and hence, gum arabic based nanoemulsions were further selected for characterization.

Table 7: Stability studies of gum arabic and plant extract based nanoemulsion with different oil ratios Stability parameters

S.NO.	Oil (%)	Plant extracts	Heating-cooling cycles	Centrifugation	Freeze-thaw	Inference
1	**1%**	*P. longum*	Clear	Clear	Clear	Stable
2		*P. niruri*	Clear	Clear	Clear	Stable
3		*B. aristata*	Clear	Clear	Clear	Stable
4		Control	Clear	Clear	Clear	Stable
5	**3%**	*P. longum*	Clear	Clear	Clear	Stable
6		*P. niruri*	Clear	Clear	Clear	Stable
7		*B. aristata*	Clear	Clear	Clear	Stable
8		Control	Clear	Clear	Clear	Stable
9	**5%**	*P. longum*	Clear	Clear	Clear	Stable
10		*P. niruri*	Clear	Clear	Clear	Stable

Table 8: Stability studies of chitosan and plant extract based nanoemulsion with different oil ratios

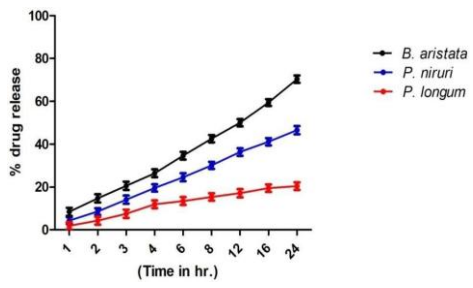
			Stability parameters			
S.NO.	Oil (%)	Plant extracts	**Heating cooling**	**Centrifugation**	**Freeze-thaw**	**Inference**

			cycles			
1	1%	*P.longum*	PS	PS	PS	Unstable
2		*P.niruri*	PS	PS	PS	Unstable
3		*B.aristata*	PS	PS	PS	Unstable
4		Control	PS	PS	PS	Unstable
5	3%	*P.longum*	PS	PS	PS	Unstable
6		*P.niruri*	PS	PS	PS	Unstable
7		*B.aristata*	PS	PS	PS	Unstable
8		Control	PS	PS	PS	Unstable
9	5%	*P.longum*	PS	PS	PS	Unstable
10		*P.niruri*	PS	PS	PS	Unstable
11		*B.aristata*	PS	PS	PS	Unstable
12		Control	PS	PS	PS	Unstable
13	10 %	*P.longum*	PS	PS	PS	Unstable
14		*P.niruri*	PS	PS	PS	Unstable
15		*B.aristata*	PS	PS	PS	Unstable
16		Control	PS	PS	PS	Unstable

PS=Phase separation

***In vitro* drug release study of formulated nanogel**

The percent cumulative drug release of xanthan gum and guar gum loaded nanogel and marketed gel was investigated by in vitro over a period of 24 h using a dialysis membrane. The in vitro release of1% Xanthan gum and 2% guar gum from the nanogel was studied. The release of drug was in following ascending order P. longum, P. niruri, and B. aristata in boththe prepared formulations. Where amount of % release in xanthan gum was 20.40% < 46.57 %< 70.34% and 21% <51.5 %< 81% release in guar gum formulations. From the study, it was concluded that guar gumnano gel formulated with B. aristata plant extract showed better drug release in comparison within 24 h. The results of drug release are reported in Fig.



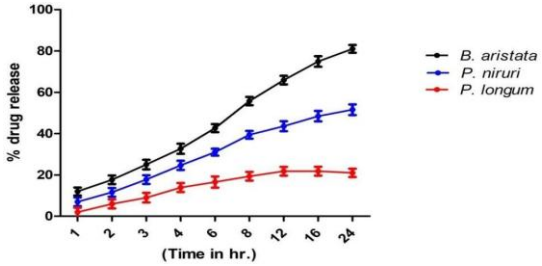


Fig 1: *In-vitro* drug release study of *P.niruri*, *P.longum* and *B.aristata*based nanogel formulations (A) Xanthun gum (B) Guar gum

4.14.1Drug Release Kinetics

Release kinetics from different plant extracts loaded with xanthan and guar gum nanogel formulationwascomparedtomarketedgelfromdifferentkineticmodelsreported in Table 10. Sustained drug release was observed during the entire period of the study showed a delayed burst release. The release kinetics was computed by fitting the release rate data to zero-order, first-order, second-order, Higuchi, and Hixson & Korsmeyer Peppas models. The best fit with the highestr^2 value was found to be shown by firstorder permeation for *B. aristata* for both xanthan and guar gum nanogel formulations which were near to 0.99, thus; release of drug from nanogel formulations followed first order release kinetic with R^2 = 0.99.

It was observed that *B. aristata* guar gum based nanogel showed better drug release with respect to marketed gel. To analyse the release mechanism of drug from nanogel formulations, the data was fit to Korsmeyer Peppas model, where then varies from 0.2 to 0.5 representing change in drug transport mechanism. It is due to the changes in polymeric ratio. Among the above formulations drug release from is best described by Quasi Fickian and Fickian diffusion release model (n<0.5 and 0.5 respectively) i.e drug release mechanism is non swellable matrix- diffusion.

Table 10: *In-vitro* drug release kinetics of different Nanogel formulations

Formulation	Zero order		First order		Higuchi		Korsmeyer-Peppas		
	K0 (intercept)	R2	K1 (intercept)	R2	KH (intercept)	R2	KKP (intercept)	N	R2
P.N(XG)	1.301	0.977	1.997	0.987	-3.851	0.937	1.213	0.302	0.973
P.L(XG)	0.766	0.932	1.997	0.938	-2.082	0.913	0.567	0.564	0.969
B.A(XG)	3.194	0.980	1.992	0.996	-4.086	0.963	3.215	0.407	0.961

P.N(GG)	2.026	0.984	1.996	0.994	-4.524	0.944	2.104	0.381	0.958
P.L(GG)	0.796	0.954	1.997	0.962	-2.685	0.924	0.592	0.204	0.982
B.A(GG)	3.666	0.987	1.998	0.993	-5.367	0.952	4.040	0.514	0.939

***In-vivo* Anti dermatophytic evaluation**

The images of antidermatophytic studies are shown in (Fig 2). In-vivo antidermatophytic treatment was started on the7th day post inoculation as the active infections were confirmed. The efficacy of guar gum nanogel were monitored on daily basis and complete eradication of *T. rubrum* was confirmed on 15th day and whereas treatment with marketed Ketoconazole was continued until 18th day. Further, to confirm that fungal infection was completely cured, areaof2cm^2 skin was scraped on the 15th day from the infected area of each animal and cultured on SDA plates and incubated. After 4 days of incubation, the plates were scored for fungal colonies and concentration of trichophytonrubrum log CFU/lesion was evaluated. The cultures treated with herbal guar gum nanogel and with Ketoconazole gel was negative for fungal growth and hair grew completely for all animals except control group where infection is given. While untreated control group showed colony forming units (CFU) log CFU 5.1.

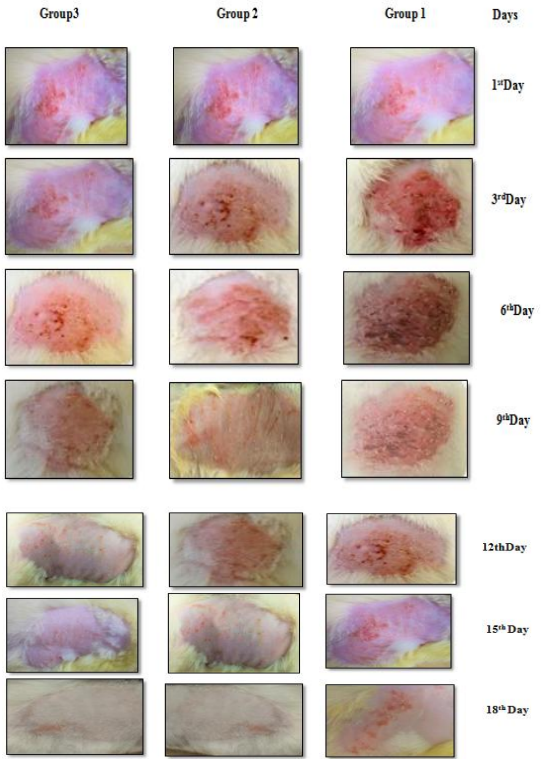
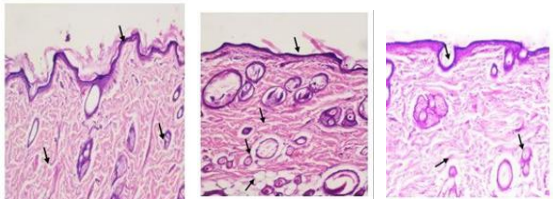


Fig 2: Schematic, diagrammatic reports of fungal cure rates during 18 days of treatment. Group 1 (Control): infected group, Group 2 (Reference): conventional ketoconazole gel, and Group 3 (Tested group): herbal nanogel

Histological Evaluation

To confirm our previous findings histological analysis of rat skin was performed by taking 1 cm^2 of animal tissue from the three treatment groups. This was done by comparing the thickness of fungal hyphae or colonization between the treated and untreated tissues. Moreover, complete eradication of the infection by *B. aristata* guargum nanogel formulation was assessed (Fig 3). Untreated infected tissues showed a thick film of fungal colonization and partial skin structure damage. However, after treatment with herbal guar gum based *B. aristata* nanogel formulation, a noticeable reduction in fungal bioburden occurred with increased intercellular gap as compared to conventional formulation of ketoconazole.



(a) (b) (c)

Fig 3: Results of histological studies of skin tissues sections after H&E staining in female wistar rat model (A) Control (infected group) (B) Skin tissue treated with conventional ketoconazole gel (C) Skin tissue treated with herbal nanogel

Stability studies

Formulation has to be remained stable for sufficient period of time. Accelerated stability studies have to be carried out to ensure stability of the optimized formulation at even adverse, variable conditions. Results of stability study of optimized nanoemulsion formulation for parameters like clog formation, phase separation, drug content and pH after keeping at 4°C, 25°C room temperature and 40°C for 3 months are tabulated in Table 11. There was no significant change in the viscosity, drug content and pH found during the stability study. It shows that the formulation passed all the tests and efficiently stable up to 3 months at variable temperature conditions

Table 11: Stability studies of optimized nanogel at different temperature conditions

Storage conditions		Parameters				
Time	Temp.	Presence of clog	Phase separation	pH	%Drug content	Consistency
1day	4°C	No	No	6.1	83.21±0.43	Good
	25°C	No	No	6.1	83.21±0.34	Good
	40°C	No	No	6.1	83.50±0.61	Good
1 month	4°C	No	No	6.3	82.95±0.50	Good

	25℃	No	No	6.3	81.48±0.42	Good
	40℃	No	No	6.4	81.50±1.20	Good
2nd month	4℃	No	No	6.2	81.45±0.90	Good
	25℃	No	No	6	81.55±0.61	Good
	40℃	No	No	6.1	81.51±1.20	Good
3rd month	4℃	No	No	6.2	81.46±0.43	Good
	25℃	No	No	6.1	82.10±1.12	Good
	40℃	No	No	6.3	82.50±0.34	Good

DISCUSSION

The phytochemical constituents are mainly responsible for the medicinal properties of the plants. Phytochemical analysis is very important to determine the quality of drug and to detect therapeutic efficacy. The phytochemical screening of methanolic extract of *P. longum* fruit showed the presence of alkaloids, phenolic compounds, carbohydrates, proteins, amino acid, flavonoid and tannins. Whereas, glycosides, phyto steroids, saponins, fixed fats and oils were found to be absent. Similar constituents were reported by the and but fixed fats and oils were present in their finding and flavonoid was absent. Methanolic extract of whole plant of *P. niruri* indicated the presence of alkaloids, phenolic compounds, glycosides, carbohydrates, proteins, amino acid, phytosteroids, flavonoid, and tannins. This finding is similar to the Shanmugam *et al*., (2014) as they reported absence of resins and glycosides. The Phytochemical screening of methanolic extract from bark of *B. aristata* yields themost promising secondary metabolites such as alkaloids, phenolic compounds, saponins, proteins, amino acid, flavonoid, tannins, glycosides, carbohydrates, phytosteroids. The finding is similar to earlier reports that revealed the presence of alkaloid, steroids, saponins, tannins, and flavonoid in the extract of the plant. These results expose that the plant has quite a number of chemical constituents, which might be responsible for the many pharmacological actions.

Nanoemulsions are proven to be drug delivery vehicles or carriers which improve the drug penetration level and drug release profile. A nanoemulsion may be a transparent or translucent emulsified system with nanometer-sized droplets which improves the bioavailability of herbal products Plant extracts, when incorporated into nanoemulsions, are thought to possess a good sort of benefits which include enhanced permeability, solubility, bioavailability, therapeutic activity, stability, improved distribution within tissues, and sustained delivery. In view of the benefits of nanoemulsions, number of studies on the incorporation of herbal drugs into these systems had been administered Now a days, a spread of methods for the preparation of nanoemulsions is described in the literature. The nanoemulsions are often prepared by both high energy and low energy methods. The interest in the low-energy methods has grown considerably because they're soft and straightforward, non-destructive, and cause no damage to encapsulated molecules Additionally, these methods are more energy-saving and thus more attractive for large-scale production

We are intended to formulate an antidermatophytic nanogel to provide local targeted action against fungal infections. The *in vitro* drug release and kinetics study was done on different kinetics model. The *B. aristata* guar gum based herbal nanogel showed higher drug release rate which could be due to the small size of nanoemulsion formulation, which permitted a faster rate of drug dissolution into the aqueous phase. The *P. longum* based nanogel exhibited slow drug release. However, slow release of the drug from the encapsulated formulation may be due to loaded drug into the nanoglobules to the buffer medium(Hussain*et al*., 2016).Thereleasekinetics fitting dataindicatedthat drugrelease followed Quasi Fickian and Fickian diffusion release model i.e drug release mechanismis non swellable matrix-diffusion**.** Results of*in vitro* release study verified that the most important factors influencing drug release from the prepared nanogels are polymer type and concentration. Polymer type proved to have a remarkable and predominant influence onthe drug release from the prepared gels followed by the polymer concentration. Many studies showed that drug release was decreased with an increase in gelling agent concentration. As the polymer concentration increases, viscosity increases. *In vitro* antidermatophytic activity of optimized herbal nanogela gainst dermatophytes was carried out and revealed that the during 72 h of incubation period, guar gumn a nogel formulated with *B.aristata* (2:1:1:1) showed significant ($p<0.05$) area of zone of inhibition as compared to *B. aristata* xanthan gumgel(1:1:1:1) against *T. rubrum*. This may be due to the small droplet size and sustained diffusion of active ingredient into the medium. Thus, formulating *B. aristata* nanogel could be an effective way to improve antifungal potency and also be helpful to counter increased drug resistance toward conventional antifungal therapies. Smaller globule size of *B. aristata* nanogel enhances the permeation of the drug and establishes the high concentration gradient on the infected areas which provides efficient dermal drug delivery. The killing of fungusis possible only if larger amount ofdrug permeates into the
skin layers and is retained for a prolonged period of time at the infected areas. Thus, *B. aristata* nanogel could be a promising form of drug delivery for the treatment of dermatophytic diseases.

In vivo antidermatophytic cure rate studies confirmed that *B. aristata* based guar gum nanogel is showing better results as it was achieved complete cure on 15th day as compared to marketed ketoconazole which was continued until 18th days. The result agreed with the previous as they prepared ketoconazole loaded nanoemulgel for the topical treatment of onychomycosis which was found to be effective against *T. rubrum* and *C. albicans* and was non-sensitizing and safe for topical use. Inthe histological analysis ofrat skin, untreated infected tissues showed a thick film of fungal colonization and partial skin structure damage. After treated with herbal guar gum based *B. aristata* nanogel there is a complete reduction in fungal bioburden with increased intercellular gap in comparison to conventional ketoconazole. This could be attributed to the extended drug release of formulation which assisted drug penetration to skin tissue and increased the duration of action of the drug and its bioavailability. *In vivo* studies had confirmed the prepared *B. aristata* nanogel retarded the growth of *T. rubrum*. At last, stability studies was carried out indicating that the *B. aristata* guar gumbased nanogel formulation is stable and found to be consistent with no change in drug content, pH values, phase separation, and transparency and efficiently stable up to 3months at variable temperature conditions. Hence, could be verypromising and innovative topical alternative for treatment of skin infections caused by *T. rubrum*and play a vital role in drug efficiency.

SUMMARY AND CONCLUSION

Fungal infection srema in a continuous and growing threat to human health. Dermatophytes are the most common agents of superficial fungal infections worldwide and widespread in the developing countries, especially in the tropical and subtropical countries like India, where the environmental temperature and relative humidity are high (Gupta et al., 2014). It has been reported that 20%–25% of human population show presence of skin fungal infections.

Dermatophytosis, commonly known as ringworm infection and T. rubrum among dermatophytes, are the most familiar species, especially in countries of tropical regions like India where dermatophytosis prevalence ranges from 36.6–78.4%. There are several effective antifungal drugs available, but their therapeutic efficacy is limited due to unfavorable physicochemical characteristics and high toxicity profiles. In search of alternate therapy, herbal plants and oils are explored as they harbour nonphytotoxic compounds and have antimicrobials and anti dermatophytic.

Today, the entire world is increasingly interested in natural medicines and there is a growing demand for plant- based medicines. Application of herbal drugs into nanotechnology is a novel approach to magnifying the solubility, absorption rate and permeation membrane of phytomedicine, which has the high bioavailability and therapeutic potential of these medicinal plants. Thus, to overcome such limitations, various novel drug delivery systems were developed. Nanoemulsion formulation a novel drug delivery system have capability to minimize these drawbacks of antifungal drugs due to their unique properties like high biocompatibility, ease of surface modification, and smaller size. Nanoemulsion based topical formulations are effective to treat fungal skin infections as they often enhance the therapeutic efficacy and tolerability of locally applied antifungal drugs.

The present study provides a novel herbal nanoformulation for the treatment of fungal skin infections. In the present study, extraction of the medicinal plants (Pipper longum, Phyllanthus niruri and Berberis aristata) and C. lanatus seed oil was carried out. After the extraction process, the extracts were used for quantitative and qualitative assessment of phytoconstituent, their antioxidant and anti-inflammatory potential. Among the selected plants Berberis aristata showed highest phenolic, flavonoid, antioxidant and anti-inflammatory content. Moreover, the GCMS profiling of the oil extracted from C. lanatus seeds identified eight main components from which ç Tocopherol is recorded as a fat-soluble vitamin E bearing potential antioxidant, antimicrobial, antifungal and anti-inflammatory activity. After that, plant extracts of Pipper longum, Phyllanthus niruri and Berberis aristata with C. lanatus seed oil based nanoemulsion formulation was prepared with different biopolymers namely sodium alginate, chitosan and gum arabic.

On the basis of physical evaluation, stability parameters and low droplet size, gum arabic based nanoemulsion with varying concentration of oils was selected to formulate into nanogel with two gelling agents i.e xanthan gum and guar gum. Nine different ratios of Xanthan gum and guar gum were formulated with five different ratios of oils i.e (1%, 3%, 5% and 10%). After that on analyzing physical evaluation, droplet size and polydispersity index characterization. Out of total 72 ratios, 36 ratios of xanthan gum and 36 ratios of guar gum it was found that 2:1:1:1 ratio of guar gum nanogel and 1:1:1:1 ratio of xanthan gum nanogel showed good

stability and least particle size ranged between 172 – 184 nm and PDI values remained between 0.26-0.35 respectively. After optimizing, the 5% ratio of C. lanatus seed oil was selected for formulation with ratio 1:1:1:1 of xanthan gum and ratio 2:1:1:1 of guar gum for formulating herbal nanogel. Out of all herbal formulations, the guar gum based B. aristata nanogel showed good results with least particle size of 206.3 nm and 0.24 PDI value as compared to other herbal nanogel. The pH was near to pH of the skin which revealed nonirritating nature of the formulation. In addition to this, in vitro drug release studies showed that the B. aristata guar gum based herbal nanogel showed higher drug release rate which permitted a faster rate of drug dissolution into the aqueous phase. The release kinetics fitting data indicated that drug release followed the Quasi Fickian and Fickian diffusion release model. Further, in vitro antidermatophytic activity of optimized herbal nanogel against T. rubrum and T. mentagrophytes was carried out. The results revealed that guar gum nanogel formulated with B. aristata showed significant zone of inhibition as compared to B. aristata xanthan gum nanogel and ketoconazole against T. rubrum.

After optimization and in vitro studies, B. aristata based xanthan gum and guar gum based nanogel was selected further for zeta potential, TEM, FESEM, spreadability, extrudability, rheological behavior and texture analysis. Furthermore, the negative value of zeta potential i.e (-23.7 mV and -29.9 mV) indicate that the prepared nanogels showed better stability. The TEM images showed the small droplet size of nanogel and these were confirmed well in accordance with the results of particle size analysis. The FESEM study revealed that the prepared nanogel was in the form of clusters and mostly uniform dispersion was found which was well dispersed in emulsion system. The spreadability and extrudability tests of nanogel observed that both nanogel formulations showed good spreadability which is required for easier topical application of the formulation.

Rheological and texture studies confirms that the B. aristata guar gum based nanogel exhibited pseudoplastic flow behavior with shear thinning. Whereas, texture analysis showed that B. aristata guar gum nanogel exhibited good results with high value of consistency, firmness and cohesiveness as compared to B. aristata xanthan gum based nanogel. Based on the preliminary studies i.e. higher stability, drug release, optimum low droplet size, characterization and in vitro analysis against antidermatophytic activity the B. aristata based guar gum nanogel showed better results and hence, was selected for the in vivo study. In vivo skin irritation study shows that the formulated nanogel was non-irritating and safe for topical use on the rat skin. In vivo antidermatophytic and histological studies confirmed that B. aristata based guar gum nanogel was significant than marketed ketoconazole. B. aristata based guar gum nanogel was achieved complete cure on 15th day as compared to marketed ketoconazole which was continued until 18^{th} days. In vivo studies had confirmed the prepared B. aristata nanogel retarded the growth of T. rubrum. In the end stability studies was carried out indicating that the B. aristata guar gum based nanogel formulation is stable and found to be consistent with no change in drug content, pH values, phase separation, and transparency for upto 3 months. Thus, our B. aristata nanogel is a safe, effective and promising formulation for the topical treatment of dermatophytic skin infections.

CONCLUSION

The present study used to formulate the antidermatophytic nanogel for topical delivery to provide efficient local targeted action with better permeation and sustained effect in spite of its low permeability and aqueous solubility, which achieved. Conversion of this nanoemulsion formulation into gel provided sustained effect with increase in the retention capacity. Formulation, being nano size efficient larger interfacial area to penetrate into the skin tissues in higher amount and less greasy, easy to spread than other topical forms such as ointments, creams etc. Hence, it is concluded that B. aristata guar gum based nanogel can be exploited in delivery of promising vehicle for the topical application of superficial skin infections. It appears that novel drug delivery systems open new opportunities for delivery of herbal drugs at right place, at right concentration, for right period of time and also increase efficacy and patient compliance. Therefore, the study postulates that the novel nanogel formulation can be proved fruitful for the treatment of antidermatophytic skin infections in near future.

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