Development, Characterization, And Evaluation Of Anti-Fungal Activity Of Nystatin Loaded Nanogel Prepared From Biodegradable Polymer

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Abstract:
One well-known fungal infection of the skin, topical candidiasis, is typically treated with traditional dose forms like cream, gel, or emulgel, which have a number of negative consequences on the skin. Novel medication delivery techniques have been explored as a means of mitigating these drawbacks. Systematic use of polymer-based nanoparticulate materials has demonstrated good skin penetration. Thus, the pathophysiology, etiology, and subsequently topical treatment of candidiasis were the primary foci of the current investigation. Using natural polymers like chitosan and gelatin, nanogel containing Nystatin has been created. Nystatin's 100 mg nanogel was created using a modified emulsification-diffusion process and was assessed for a number of factors. Out of the seven nanogel formulations denoted as F1 through F7, F1 has been chosen as the model formulation due to its high percentage of gelatine and chitosan (83.86 and 18.16, respectively). This was because the formulation F1 was determined to be optimal and stable in accordance with the ICH stability guidelines. The greatest release, or roughly 88.57%, was likewise demonstrated using Nystatin nanogel formulation F1. The prepared nanogel was shown by XRD to be crystalline. In conclusion, topical preparation and nano size are the two primary benefits of the Nystatin nanogel drug delivery systems. One theory is that the most effective treatment for fungal skin disorders might involve the use of nanogel.

Keywords: Nanogel, Anti-fungal, Nystatin, Biodegradable Polymer, Gelatine, Chitosan, etc.

Introduction

Nystatin, sometimes known as Nys, is a widely used antifungal medication. Strains of Streptomyces noursei produce this membrane-active polyene macrolide [1]. Since Nys has been developed in liposomes and intralipid formulations for intravenous delivery [2, 3], interest in it has recently returned [4]. This is because the formulations aim to prevent toxicity associated with systemic exposure. The primary indication for Nys remains the treatment of fungal infections of the skin and mucous membranes, notwithstanding these efforts [5].

Fungal infections are a frequent cause of illness. These infections include dermatitis, dermatophytosis, intertrigo, candidiasis, and congenital candidiasis. Oral or parenteral dose forms are used to treat many fungal diseases, and topical formulations are only partially effective in treating certain conditions. Comparably, nanoparticles are currently being developed and evaluated for parenteral and oral administration; just a small amount of research has been done for topical administration. Drug delivery systems based on nanoparticles have gained a lot of attention for their ability to deliver medications to difficult-to-reach locations in the body. As a result, many methods for the site-specific targeting of these medications have been documented in the literature to address these problems. These nanoparticle systems' wide surface area and tiny size, which promote cellular absorption, are two of their main advantages [6–8].

In medicine and formulations research, nanotechnology plays a significant role, especially in protecting against early drug degradation and developing potential targeted and site-specific delivery systems that increase absorption into particular tissues and improve intracellular penetration with reduced drug toxicity and increased efficacy. Since they can load both hydrophobic and amphiphilic medications and have a high water content, modify drug release, and are biocompatible, nanogel—also referred to as nanoparticulate hydrogels—are being considered as possible drug delivery vehicles. The first stage is to create a gelatine-based nanogel using one of three methods: inverse miniemulsion, nano precipitation, and two-step desolation. Rheological behavior, surface morphology, particle size, zeta potential, drug loading, and release characteristics of the loaded antifungal medicines are among the most crucial aspects of nanogel characterization that must be carried out [9, 10].

Due to their extremely small size and ability to provide successful targeted drug delivery—particularly for medications that are ineffectively given by conventional delivery methods—nanoparticles are currently the subject of intense research in the drug development process. A previously unreported antifungal medication called miconazole has been put into a gelatine nanogel for topical or cutaneous administration. The majority of antifungal medications come in parenteral or oral formulations, both of which have harmful side effects. Preparing nanogel with the highest possible drug loading for efficient skin penetration was the aim of this research project [11].

Natural and thought to be more friendly and benign in nature, gelatine is a biodegradable polymer. For incorporation into gelatin-based nanogel, antifungal drugs were investigated. While topical administration is the current project's goal to avoid potential side effects and exclusively target delivery to the infected spot, antifungal medicines are typically administered orally or parenterally [12].

Material and Methods (23-29)

Materials

Nystatin was purchased from Manus Aktteva Biopharma LLP, while gelatin, chitosan, and poly (ethylene glycol) (PEG, Mn of 10,000 g/mol), as well as methacrylic acid, dichloromethane, and ethanol, were sourced from Sigma Aldrich in the United States. The supplies for PEG 6000, acetone, and acetonitrile came from Musaji Adam and Sons. Except when otherwise noted, all chemicals and reagents were used unaltered from when they were obtained.

Preparation of Antifungal drug loaded nanogel

The modified emulsification-diffusion process was used to create the nanogel for the antifungal medication. 20 ml of ethyl alcohol containing gelatine that has already been broken down by constant stirring and 100 mg of Nystatin were combined. The 40 ml of the aqueous phase were mixed with the produced organic phase dispersion. At a speed of about 8000 rpm, methacrylic acid (MAA) was added to the aqueous phase in a high-speed homogenizer. The organic solution was added to the MAA-containing aqueous phase. The resulting dispersion was then sonicated for 15–20 minutes after being constantly agitated at 10,000 rpm for 15 minutes. In order to facilitate the easy diffusion of the organic phase into the aqueous phase, double distilled water (DDW) was also added to the produced dispersion while it was being continuously stirred for 45 minutes. The nanodispesion was created as a result of this. Lecithin, a gelling ingredient, was added to the generated nanodispesion to create gels. The procedure was carried out while being stirred continuously. The prepared nanogel pH was raised to 7. Seven different formulations were prepared by changing the ratio and percentage of polymers as shown in table 1 [13].

Table 1: Composition of different nanogel formulations

Sample No.	Drug (Nystatin)	Gelatine/100 g solution	Gelatine/Chitosan (Wt%)	Polyacrylic acid/100 g of Ge/PVP (Wt. %)	MAA/100 g solution
F1	100 mg	14.5	82.85/17.15	3.8	2.660
F2	100 mg	16.5	84.61/15.39	3.8	2.964
F3	100 mg	19.0	86.36/13.46	3.8	3.344
F4	100 mg	16.5	82.50/17.50	3.8	3.040
F5	100 mg	16.5	80.48/19.52	3.8	3.116
F6	100 mg	16.5	78.57/21.43	3.8	3.192
F7	100 mg	16.5	84.61/15.39	3.8	2.730

The Prepared nanogel were evaluated by following parameters [14-22]

FTIR study

To determine compatibility in all the formulations, Bruker FTIR (ATR) spectroscopy was used to conduct infrared absorption spectroscopy on medication and Nanogel formulations.

Scanning Electron Microscopy (SEM)

SEM was used to examine the shape and surface morphology of the Nanogel created with optimal settings.

Rheology

To assess the viscoelastic properties of the Nystatin loaded nanogel Brookfield Rheocalc V 32 Rheometer was used. Data analysis of the nanogel was generated by Rheocalc software. Nanogel was studied for shear rate at an increasing order of 0 to 50 D [1/s]. All the readings
were taken in triplicate at 25ºC.

Particle size

The nanometre range was identified by the particle size study for the Nanogel. The homogenization time and gelling agent concentration both had an impact on the size of the nanoparticles.

Homogeneity

When the gels had been placed in the container, the homogeneity of the generated nanogel was checked visually. They underwent examinations to check for aggregates and to see how they looked.

pH

Using a digital pH metre, the pH of several nanogel compositions was assessed.

Spreadability
Glass slide and wooden block measuring tools were used to determine it. Weights weighing about 20 g were placed to the pan, and the amount of time it took for the upper slide (movable) to fully separate from the fixed slides was recorded. Next, spreadability was determined using the following formula:
S = M.L / T
Where,
S = Spreadability, M = Weight tide to upper slide, L = Length of glass slide
T = Time taken to separate the slide completely from each other

Extrudability
Collapsible aluminium tubes were filled with the mixtures. The extrudability of formulations was tested by forcing the tubes to extrude a 0.5 cm gel ribbon in 10 seconds.

Drug content studies
In a 100 ml standard flask, the quantity of gel equal to 100 mg was taken. After adding 25 ml of ethanol and 25 ml of 7.4 pH phosphate buffer and shaking for about 30 minutes, 100 ml of the 7.4 pH phosphate buffer was added. After filtering the solution, 5 ml of the filtrate was removed and made into 100 ml of phosphate buffer with a pH of 7.5. A specified wavelength was used to assess the solution's absorbance, and the amount of medication present was estimated.

Viscosity - At 25°C, the viscosity of the formulations (gel) was measured in cps using a Brookfield viscometer with spindle number S-96 rotating at 1 rpm. Each formulation was measured three times, with the average values being computed.

In vitro drug release
Franz diffusion cells with porous membranes were used for in-vitro drug release investigations to analyse the drug's release pattern from the manufactured nanogel. The membrane's surface was treated with each nanogel formulation. The 1% w/v phosphate buffer saline with a pH of 7.4 was pre-filled into the receiver compartment of the Franz diffusion cell and agitated at 350 rpm. The Franz diffusion cell's temperature was kept at 37 °C. The samples were taken at intervals of 0, 1, 2, 4, 6, 8, and 24 hours, respectively. After every moment, 0.5 ml of the sample was taken and replaced with the same volume of fresh buffer solution.

Stability studies
The stability research followed ICH guidelines. All the Nystatin loaded nanogel samples were individually placed in glass vials and stored for three months at temperatures of 30°C and 65% relative humidity, or ambient settings, and 40°C and 75% RH, or accelerated conditions. Following that, the vials were observed and the nanogel's appearance and clarity were evaluated.

In vitro antifungal activity
With a few adjustments to the procedure described before, Nystatin -loaded nanogel was tested for its antifungal activity against Candidiasis albicans. A mean inhibition zone (MIZ) calculation was made. To evaluate the antifungal activity of a medicine, its value was utilised as an indication.

Fungal inoculum
A Candidiasis albicans culture was obtained and left in the lab overnight. It was diluted with sterile 0.9% saline solution after 12 hours to produce cultures that contained 106 colony-forming units/mL of the bacterium.

Plate diffusion method
The placebo sample and the Nystatin loaded nanogel were compared. 20 mL of dextrose agar was used to create the petri dishes, and the candidiasis inoculum (1% w/w) was seeded. The candidiasis inoculum was injected into the petri dishes in a volume of 200 L. Following inoculation, the plates were left to dry at room temperature for 30 minutes. After cutting the wells, 75 mg of Nystatin loaded nanogel were added to each one. The plates with Nystatin loaded nanogel wells were kept at 36°C for one day. The microorganism's inhibition zone around the wells was measured and the results estimated. Every measurement was taken three times.

In-vivo studies
The in-vivo skin deposition investigation was conducted using Nystatin loaded gelatine-based nanogel formulations (2%w/w) in comparison to a control nanogel. With the use of IP of cocktail anaesthesia, which contained xylazine (10 mg/kg) and ketamine HCL (90 mg/kg), female rats weighing between 250 and 300 g were put to sleep. With the aid of a razor, the rat's dorsal region's skin was removed. To verify that no skin was being removed, the razor was run from the tail towards the head while the hairs were observed. The rat's skin was then treated with appropriately weighed nanogel containing antifungal medication and a blank. The rats were split into two groups of five each, and samples were taken at intervals of one hour, two hours, six hours, twelve hours, and twenty-four hours. The skin of the slain animals was

carefully removed from the dorsal region after a certain amount of time had passed in order to check for drug deposition in the skin. Skin was pre-treated in order to extract the drug as has been researched, and the substance was then examined.

In-vivo antifungal activity of the drug loaded nanogel

The method described in was used to test the antifungal activity of generated Nystatin loaded gelatine-based nanogel, with a few minor modifications.

Preparation of the animals

Female albino mice weighing 110-140 grammes were housed in separate cabins and given food and water. All types of hair were removed from the animal paw. 48 Hair removal cream was used to completely remove all hairs before the candidiasis strain was given.

Preparation of candida and mice infection

Provided strain of Candidiasis albicans, which was cultivated in YPD broth, which contained peptones (20 g/L), dextrose (25 g/L), and yeast extract (12 g/L). The following day, this culture was added to freshly made YPD broth and continually agitated for two to three hours. These procedures assisted in transforming candidiasis into false hyphae that could cause cutaneous infection. A butter paper that had previously been adhered to aluminium foil was used to hold candidiasis albicans. It was then placed on the mice's right paw. As a reference, the left paw was used to determine if an infection had occurred or not. It was wrapped around the paw for seven days.

Treatment of fungal infection - After removing the aluminium foil holding the Candidiasis albicans strain from the animal's paw, which took 48 hours, the diseased paw began to be treated. Four mice each made comprised one of the two groups into which the mice were split. The second group of mice acted as a control group while the first group of mice received treatment with a medication that contained nanogel. After receiving the final dosage of medication, all the animals were euthanized 24 hours later. Skin lesions that were infected were removed and preserved in sterile saline. To release Candida albicans cells from skin samples into sterile saline solution, skin samples were allowed to homogenise. After that, it had been plated onto YPD agar and submerged in diluted saline. CFU numbers were measured after plates had been incubated at 35°C ± 1°C for 24 hours.

Result and Discussion

Physical characterization of nanogel

A transparent nanogel with good consistency, spreadability, transparency, and flow properties was visible in the generated formulations. It was noted that the distribution of the particles was uniform. Additionally, it was noted that both the polymer and the medication had homogeneous dispersion.

Rheology

The rheological behaviour of each of the generated Nystatin nanogel formulations was examined. The spreadability of the formulations and their contact time are intimately related to their rheological behaviour. The graph in the figure shows a relationship between the nanogel sheer stress and shear rate that illustrates the pseudo plastic system's thixotropic behaviour. Shear stress increased with yield value as the nanogel shear rate was raised, i.e., up curve, indicating its pseudo plastic behaviour as seen in fig. Additionally, shear stress fell proportionally as the shear rate of the nanogel was reduced (up curve), revealing the thixotropic features of the material.

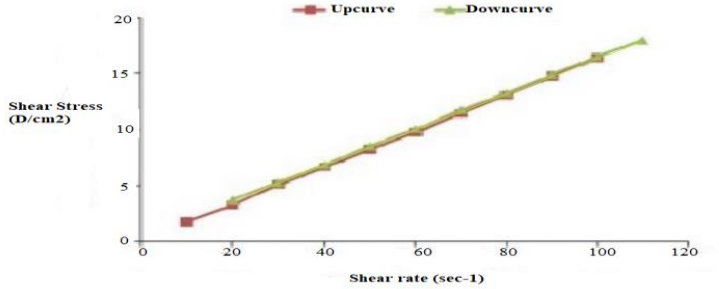

Fig. Rheology of Nystatin nanogel showing correlation between shear stress and shear rate

Particle size analysis

Using a Zetasizer particle size analyser, the size of the Nanogel's particles was determined. The size of the prepared nanogel was in the nm range. The size ranges of the generated nanogel were between 278 nm and 401 nm, which indicated that the formulations had distinct particle size distributions, as shown in the following table.

Table: Particle size analysis of Nanogel containing Nystatin

Formulation code	Particle Size Analysis
F1	**278 nm**
F2	308 nm
F3	289 nm
F4	323 nm
F5	365 nm
F6	387 nm
F7	401 nm

Homogeneity

All the gel formulations (F1–F7) displayed excellent homogeneity and lacked lumps. The presence of particles, uniformity of the gel, aggregates, foreign materials, and phase separation were not found in the gels, which were also determined to be transparent. The results are displayed in the table below.

Determination of pH

The pH of various formulations, ranging from F1 to F7, was displayed in the table. Depending on the polymer to medication ratios in each formulation, the pH fluctuates.

Spreadability

Dimensions of spreadability for various formulations F1-F7 demonstrated good spreadability, i.e., the gel is simple to spread. Table displays the findings.

Extrudability

An effective gel composition should easily extrude from the container. All formulations were determined to have good extrudability. Table displays the outcome.

Drug content - The medicine content for each formulation, ranging from F1 to F7, is listed in the table below. There are relatively minor variations in the medication content of each formulation. Therefore, in this instance, the influence of polymer ratios is less important.

Viscosity

In order to test the viscosity (in cps) of all Nanogel formulations, a cone attached to a holding rod was dropped from 10 cm onto the centre of a glass cup containing Nanogel. This was done using a Brookfield viscometer. Table displays the findings.

Table: Evaluation of formulated batches of Nanogel

Formulation code	Homogeneity	pH	Spreadability (cm)	Extrudability	Drug Content	Viscosity (cps)
F1	**Homogenous**	**6.8**	**3.6**	++	**91.16 %**	**3529**
F2	Homogenous	6.2	2.6	+	83.17 %	3460
F3	Homogenous	6.1	3.3	+	87.73 %	3357
F4	Homogenous	6.4	3.0	++	85.76 %	3269
F5	Homogenous	6.1	3.5	++	84.46 %	3499
F6	Homogenous	6.2	2.7	+	81.25 %	3296
F7	Homogenous	6.1	2.9	++	86.68%	3502

+ Satisfactory ++ Good

In vitro drug release

The discharge of Nystatin from the prepared nanogel was watched. After 24 hours, the Nystatin nanogel formulations F1 exhibited the maximum release, or approximately 88.57%, whereas the formulations F7 showed the least release, or roughly 55.36%. It was discovered during in vitro drug release tests that every formulation adhered to first order release kinetics. Figure 2 depicts the drug release from each of the seven formulations.

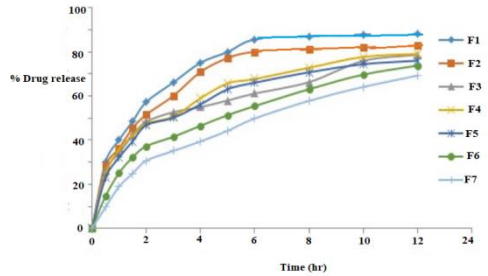

Fig. *In vitro* drug release of all form of Nystatin loaded nanogel in phosphate buffer pH 7.4

Scanning electron microscopy (SEM) of nanogel

SEM was used to examine the surface morphology and form of the nanogel that had been created. The synthesised nanogel was relatively round and oval, as seen by the SEM pictures. As shown in figs. a, b, and c, the SEM images were captured at various resolutions, with scales of 200 nm, 500 nm, and 1 m. The nanogel surface has a smooth texture. As seen by the SEM, the particle size distribution is also in nanometres. Images captured by a scanning electron microscope (SEM) indicate a generally uniform dispersion of Nystatin throughout the nanogel and some of the generated nanogel was seen in the form of clusters. In fig., SEM spectra at various scales are displayed.

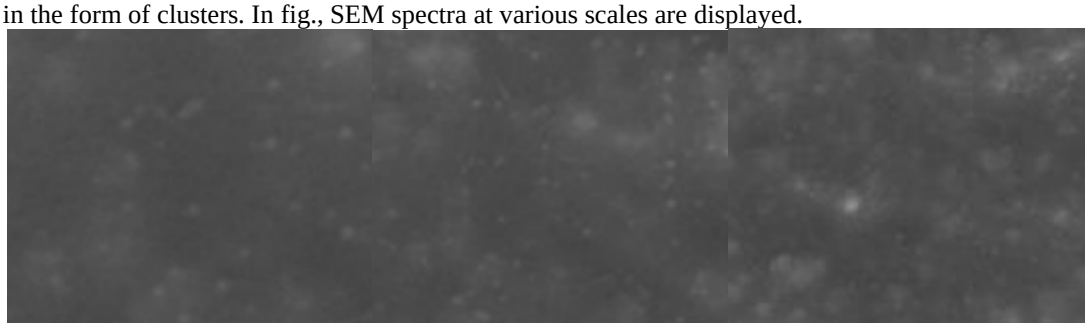

Fig. SEM image of Nanogel F1 at a) 200 nm scale, at b) 500 nm scale and at c) 1 µm scale

Fourier-transform infrared spectroscopy (FTIR)

There was no considerable change in the positions of characteristic absorption bands and bonds of various functional groups present in the drug. This observation clearly suggests that the Nystatin shows no prominent change in its characteristics even in its physical mixture. The results of FTIR spectra indicated the interaction between drug and polymer. It showed that Nystatin was compatible with chitosan.

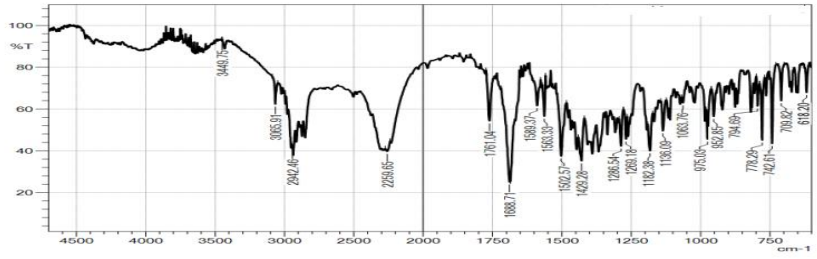

Fig. FTIR spectra of optimized nanogel (F1)

Differential Scanning Calorimetric analysis (DSC)

The DSC thermogram of Nystatin loaded nanogel formulation batch F1 are shown in following figure.

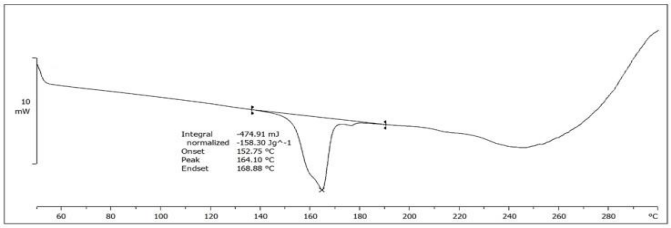

Fig. DSC thermogram of Nystatin loaded nanogel formulation batch F1

X-Ray Diffraction (XRD)

If the nanogel loaded with Nystatin formed, the diffraction pattern of the drug was unique from the superimposition of each component. To investigate the differences between the solid state and the gel generated, X-ray diffractometry can be helpful. By contrasting the various peak heights in the binary system's diffraction pattern with the reference, crystallinity has been deduced. Figures a, b, and c, respectively, illustrate the X-ray diffraction patterns of produced nanogel, gelatine, and Nystatin. As can be seen from the X-ray diffractogram of Nystatin, the presence of multiple strong peaks at the following diffraction angles (2) of 14.3°, 16.3°, 21.7°, 24.8°, 27.6°, and 30.1° indicates that the medication is present in a crystalline form. Peak nanogel intensity dropped, indicating that Nystatin's crystallinity had been lost. Most likely, it was brought on by the usage of amorphous gelatine.

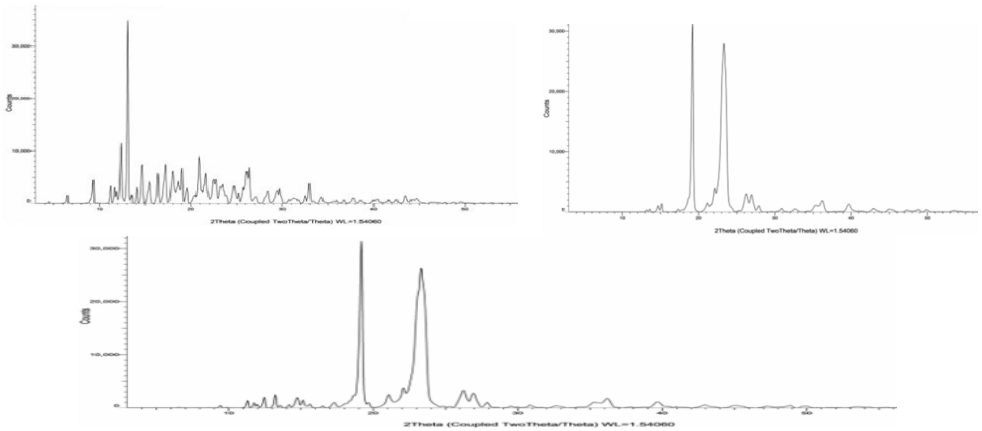

Fig. a: X-ray diffraction Spectra of Nystatin, b X-ray diffraction Spectra of Gelatine and c X-ray diffraction Spectra of prepared nanogel (F1)

Stability Studies

All seven produced formulations of nystatin nanogel had their average particle sizes evaluated during stability testing in accordance with ICH guidelines. Both clarity and look were unchanged. It was almost identical to how it had been at zero time. During the duration of ambient storage, there was no change in the nanogel's particle size. However, under accelerated storage circumstances, the nanogel's particle size grew at the end of the first, second, and third months, respectively. This demonstrated that as storage temperature was raised, particle size increased.

In-vitro antifungal activity

Numerous skin conditions have been linked to Candidiasis albicans. Candidiasis albicans was employed as a reference standard during the research to evaluate the in vitro antifungal activity of Nystatin loaded nanogel. After applying Nystatin nanogel (2% w/w) to the wells, the mean diameter of the mean inhibition zone (MIZ) of the plates was measured. When compared to a placebo where no Nystatin was used (0.42 ± 0.05), Nystatin loaded nanogel (2% w/w) demonstrated relatively good antifungal activity (35.34 ± 0.80). The increased physicochemical properties of Nystatin following its integration in the nanogel system were validated by the higher in vitro antifungal activity of Nystatin loaded nanogel as compared to blank, or having no antifungal drug.

***In vivo* Studies**

***In vivo* skin deposition studies**

To ascertain the amount of Nystatin deposited in the skin at various time intervals, Nystatin loaded gelatine based nanogel formulations were examined. The fact that each skin-specific preparation also demonstrated quick skin penetration was advantageous. The following table shows the outcome for the in vivo skin deposition of Nystatin-loaded nanogel (mean ug ± SEM).

Table: In-vivo skin deposition study representing amount of Nystatin deposited in the skin at various time intervals after single topical application of Nystatin loaded nanogel

Time (hour)	Formulations (µg deposited)
1 h	**70.2 ± 1.9**
2 h	62.3 ± 2.8
6 h	51.5 ± 1.8
12 h	25.5 ± 4.7
24 h	20.8 ± 1.5

This table showed how quickly Nystatin-loaded nanogel was deposited into the skin, with 70.2 ± 1.9 g of Nystatin from the gelatine-based nanogel present after 1 hour.

***In vivo* antifungal activity**

Female albino mice were used to compare the anti-fungal activity of a gelatine-based nanogel containing 2% w/w Nystatin to that of the other controlled group of animals. Mice's paws were infected with fungus, and the Nystatin-loaded nanogel antifungal activity was then determined through a quantitative study of the fungal infection in terms of CFUs discovered in the lesion of the infected paw following the course of therapy. Nystatin-loaded nanogel in vivo microbiological activity was contrasted with an untreated negative control. The mice were slaughtered, their skin was removed, and it was homogenised in sterile saline after the three-day treatment period. The CFUs of Candidiasis albicans were measured following homogenization. According to in vivo anti-fungal action, as shown in fig., the Nystatin-loaded nanogel demonstrated log CFU of 2.06 ± 0.35 while the untreated group demonstrated log CFU of 5.14 ± 0.05.

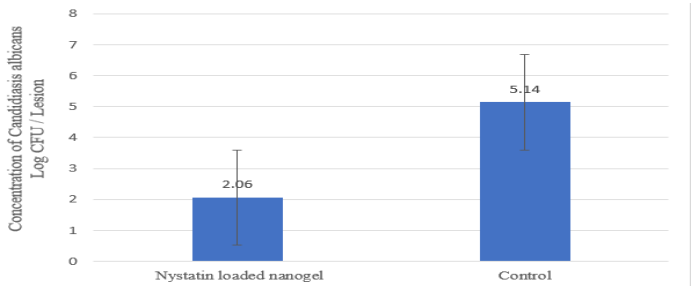

Fig. *In vivo* antifungal efficacy of Nystatin loaded nanogel as compared to untreated group (control) after three successive days of single application per day.

Conclusion

The adapted emulsification-diffusion approach was utilized to effectively create topical nanogel laden with Nystatin. According to examinations using scanning electron microscopy, the generated nanogel particles had a spherical shape, but drug crystalline structure was absent from the final nanogel formulation, according to DSC investigations. The medication and polymer were both integrated into the produced nanogel, according to FTIR analysis. The effectiveness of the Nanogel topical distribution was demonstrated by its physicochemical properties. The produced nanogel stopped Candidia albicans from growing, as demonstrated by its antifungal activity both in vitro and in-vivo.

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