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Design of different size nanospheres for skin application as an Ointment - Ketoconazole

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ABSTRACT: The development of nanotechnology has revolutionized drug delivery, offering innovative solutions to enhance the efficacy of topical treatments. This study focuses on the design and characterization of nanospheres of varying sizes intended for skin application as ointments. Various nanosphere formulations were synthesized using biocompatible polymers and surfactants. The size of the nanospheres was systematically varied, spanning from nanometers to micrometers, by adjusting formulation parameters such as polymer concentration and emulsification techniques. The choice of nanosphere size was found to significantly influence their physical properties and potential applications.This research lays the foundation for the development of customizable nanosphere-based ointments, enabling tailored drug delivery strategies for skin treatment.

KEYWORDS: Nanospheres, Polyhydroxybutyrate, *Bacillus spp*., nanoprecipitation Ketocanozole, Antifungal ointment

I. INTRODUCTION

Numerous carrier systems have been extensively studied in the last 30 years with the aim of controlling drug release and enhancing the efficacy and selectivity of formulations. Controlled drug release systems are intended to deliver an appropriate reaction at the needed site of action for extended periods of time, hence improving treatment. These systems can be supplied via a variety of methods, including intravenous, oral, intraperitoneal, intramuscular, subcutaneous, and cutaneous administration. Controlled drug releasing techniques can be achieved by Nanosphere. Nanospheres have small particle sizes, making them suited for systemic, local, and oral administration [1]. Typically, biodegradable and biocompatible polymers are used to create most nanospheres. They serve as a delivery method to improve the drug's entrapment and release.The majority of nanospheres are produced with synthetic, biocompatible, and biodegradable polymers. Nanospheres can be delivered orally, locally, and systemically because of their small size. Nanospheres are uniform spheres in which an active ingredient or medicine is disseminated and either adsorbed on the surface or trapped inside a polymeric matrix structure by the solid sphere. The nanospheres are between 100 and 1000 nm in size [1], It is made using the phase inversion method, solvent displacement, solvent evaporation, and microemulsion polymerization techniques [2]. Using hydrophilic gels to disperse polymer-based nanoparticles can further enhance medication delivery to the skin. The gel can help the carriers spread evenly throughout the matrix, lengthen the time they are in touch with the skin, and deposit more carriers there, which will improve the payload's ability to penetrate the skin.

1.2 NANOSPHERE

Nanospheres are spherical particles with a diameter of 10-1000 nanometers. They can be made from a variety of materials, including polymers, lipids, and metals. Nanospheres have a wide range of applications in biology. Nanospheres can penetrate through small capillaries, across physiological barriers and incorporated into cells. Therefore, in recent years, significant effort has been devoted to developing drug-delivery nanospheres for treating various diseases such as cancer, due to the potential for more targeted localization in tumors with active cellular uptake [3]. Nanospheres are usually designed as short-acting delivery vehicles and administered via intravenous, intramuscular, subcutaneous, oral, nasal, ocular or transdermal routes, either fluidized with a liquid carrier or as a solid powder[4]. nanospheres were incorporated into larger spheres by flocculation, spray drying, or other means, so that the spheres have a desired size to target a specific disease site, such as that in pulmonary drug delivery [5] Nanospheres can also be microencapsulated using enteric coatings in order to control release and degradation in vivo [6]. It has also been reported that particle shape might also have a significant impact on polymer degradation and, therefore, drug release profiles [7]. There are several techniques available for the preparation of drug-loaded micro- and nanospheres, such as emulsion-solvent evaporation/extraction methods, solvent displacement, phase separation, self-assembly, rapid expansion of supercritical fluid solution, and spray drying.

1.3 BIOPOLYMERS

Biopolymers are the organic substances present in natural sources. Large macromolecules made up of numerous repeating units are known as biopolymers [8]. As per the IUPAC definition, a macromolecule defines a single molecule [9]. The biopolymers are found to be biocompatible and biodegradable, making them useful in different applications, such as edible films, emulsions, packaging materials in the food industry, and as drug transport materials, medical implants like medical implants organs, wound healing, tissue scaffolds, and dressing materials in pharmaceutical industries. The main focus of this review is to provide a piece of knowledge about biopolymers and their uses in the food and medical industries. The most prevalent macromolecules are biopolymers, which comprise nucleic acids, proteins, carbohydrates, lipids, and giant non-polymeric molecules like lipids and macrocycles, the most frequent macromolecules [10]. Plastics, synthetic fibers, and experimental materials, such as carbon nanotubes, are examples of synthetic macromolecules [11]. In addition to repeating units of nucleic acids, saccharides, or amino acids, their molecular backbones may contain a variety of chemical side chains that contribute to the functions of the molecules. Polylactic acid (PLA) and polyhydroxyalkanoates (PHAs) are two examples of biopolymers found in microorganisms or genetically modified organisms utilizing traditional chemical methods. These include polysaccharides from cellulose and proteins from collagen or milk. The biotechnological synthesis of biopolymers with customized qualities suited for high-value medical applications, such as tissue engineering and medication delivery, is made possible through the genetic modification of microorganisms.

1.4 POLY-HYDROXYBUTYRATE AS BIOPOLYMER

Polyhydroxybutyrate (PHB) is a biodegradable polymer that is one of the most commonly observed forms of PHAs, as it is composed of packed monomers of R-3-hydroxybutyrate (R-3-HB) [12]. PHB is the only type of polymer that is fully biodegradable in nature. Bacteria can synthesize PHB as inclusion bodies that accumulate as reserve material when their growth is subject to several different stress conditions [13]. This polymer exhibits properties that are similar to several synthetic thermoplastics, including polypropylene. The advantages of these types of biodegradable plastics are that they are useful for extensive applications and can be produced on a mass scale. This will enable us to replace the petroleum-

based plastics that are currently used by the industry. The high production costs of PHB in comparison with plastics derived from petrochemicals are one of the major problems for the extensive production and commercialization of this product. A great deal of effort has been put into reducing the cost of producing PHB in recent years by utilizing strategies such as developing efficient bacterial strains and optimizing the fermentation and recovery processes [14]. It has been suggested that, based on most of the reports regarding the production of PHB, the carbon substrate cost is one of the major contributors to the overall production costs of PHB [15]. It is for this reason that the choice of an efficient carbon substrate is one of the most important steps in determining the final cost of the product. Alternatively, a renewable, economically feasible, and most readily available carbon substrate can be selected and used for both microbial growth and the efficient production of PHB by microorganisms [16].

1.5 BACILLUS SPP.

Bacillus, a genus of Gram-positive bacteria, is characterized by its rod-shaped morphology and belongs to the phylum Bacillota, boasting a roster of 266 officially recognized species. Bacillus species exhibit adaptability in terms of oxygen utilization, encompassing both obligate aerobes, which rely on oxygen, and facultative anaerobes, which can thrive in its absence. In laboratory cultures, Bacillus species yield a positive catalase test result when oxygen has been utilized or is present. Bacillus spp have the ability to produce biopolymers. Though these carbonosoms accumulation has been investigated in various bacterial isolates, Bacillus species are extensively studied in PHAs world since the exploration of poly-β-hydroxybutyrate (PHB) in the cytosol of Bacillus megaterium by the French Lemoigne, in 1926 [17]. Some Bacillus species have been reported to produce as much as 90% (w/w) PHAs of dry cells during nutrient imbalance [18]. Bacillus species becoming model organisms in industry and academic world attributed primarily to its genetic stability [19]. Apart from higher growth rate compared to other bacteria, the use of Bacillus species to produce PHAs is advantageous over others due to the absence of lipopolysaccharides external layer in them which makes PHAs extraction much simpler [20]. Bacillus species are also capable of producing PHAs copolymers utilizing the relatively simple, inexpensive and structurally unrelated carbon sources. Moreover, the isolates possess the ability to secrete a number of hydrolytic enzymes that can be exploited for cost affordable PHAs production by utilizing, for instance, agro-industrial and other waste materials [21]. The major drawback of Bacillus species in PHAs production is their sporulating nature. Practically the fact of sporulation and deposition of PHAs granules provoked due to stress factors [22]. To overcome the predicament research on pilot scale PHB productions by B. cereus in the media that depresses sporulation, under acidic pH and potassium deficiency conditions. These pores over strategies not only inhibit spore formation in Bacillus but also can enhance the PHAs productivity. Several studies of PHAs are dealing mostly with upstream and downstream processes, its applications and with genetic modifications or mutations to increase the yield [23]. Now these expertises become an impediment, being economically infeasible to market. This review summarizes these recent trends in PHAs production by Bacillus species as an effort to provide direction and leads to future research and development towards the growing quest for biodegradable plastics, one more critical step ahead towards an eco-sustainable development.

1.6 KETOCONAZOLE AS DRUG

Ketoconazole is the member of the imidazole class that is currently used in the treatment of systemic infections. Ketoconazole is classified in the Biopharmaceutics Classification Scheme (BCS) as a class II drug, since it has a high permeability and poor solubility. Ketoconazole is best absorbed at highly acidic levels, so antacids or other causes of decreased stomach acid levels will lower the drug's absorption. Absorption can be increased by taking it with an acidic beverage. It is very

lipophilic and tends to accumulate in fatty tissues. Ketoconazole works principally by inhibiting the enzyme cytochrome P450 14-alpha-demethylase (P45014DM). Many solubilization techniques have been described that either changes the nature of solvent environment (co-solvents systems, emulsions, micellization) or the chemical identity of the desired solute (salt formation, prodrugs); however, in comparison drugs into hydrophilic carriers is an alternate option for improving the drug bioavailability. Such dosage forms are referred to as solid dispersions [24]. Ketoconazole is prepared by nanocrystallization, solid dispersion, hydrotropy and Inclusion complex formation technique. In vitro release profiles were evaluated and compared with standard ketoconazole [25].

1.7 NANOPRECIPITATION

The most straightforward approach for creating a polymeric nanosphere containing drugs involves a technique known as the solvent displacement method, also referred to as the nanoprecipitation method. This method was pioneered by Fessi and colleagues [26]. In essence, it relies on two solvents that can mix with each other. Typically, both the polymer and the drug need to be dissolved in the first solvent, which is referred to as the solvent, but they should not be soluble in the second solvent, known as the non-solvent. However, it's worth noting that in addition to solvents that can mix with water and non-halogenated solvents, immiscible solvents such as dichloromethane can also be employed. During nanoprecipitation, the polymer rapidly undergoes desolvation, leading to the formation of nanospheres when the polymer solution is introduced into the non-solvent. Once the polymer-containing solvent has diffused into the dispersing medium, the polymer promptly precipitates, resulting in immediate entrapment of the drug. The nanoprecipitation technique is known for its simplicity, minimal complexity, low energy requirements, and broad applicability in producing well-defined nanospheres [27]. Nevertheless, the inclusion of a stabilizer is crucial to prevent particle aggregation and ensure the stability of the nanospheres throughout the nanoprecipitation process [28].

1.8 ANTIFUNGAL ACTIVITY

The well diffusion method, also known as the agar well diffusion method or Kirby-Bauer method, is a widely used microbiological technique for evaluating the antimicrobial properties of substances such as antibiotics, plant extracts, or chemical compounds. This method involves the cultivation of microorganisms, typically bacteria or fungi, on nutrient agar or Mueller-Hinton agar plates. Once the microbial culture has been evenly distributed across the agar surface, wells are created in the agar using sterile tools. These wells are then filled with the substance being tested for antimicrobial activity. After an appropriate incubation period, during which the microorganisms have had the opportunity to grow, the plates are examined for zones of inhibition – clear areas surrounding the wells where microbial growth has been inhibited by the tested substance. The size of these zones of inhibition is indicative of the effectiveness of the substance against the specific microorganism.

II. MATERIALS AND METHODS

2.1 COLLECTION OF SAMPLE AND ISOLATION OF BACTERIA

Soil samples were collected from different places in order to isolate PHB-producing bacteria. In 9 ml of sterilized water, 1g of soil samples were added. In the following serial dilution, nutrient agar plates were streaked with the dilutions(10^{-7}). Incubation for 24 hr at 40^{o} C to isolate *Bacillus spp*. Purified strains were maintained at 4^{o} C on a nutrient agar slant and purified. The constituents of nutrient agar plates are Beef extract , Peptone , Sodium chloride and Agar [29].

2.2 SCREENING OF PHB PRODUCING *BACILLUS SPP* BY SUDAN BLACK TECHNIQUE

A Sudan black staining technique has been implemented to screen the isolates for the presence of PHB granules. The isolated bacteria

were grown in the LB plate and incubated for 48 hr at 37° C. After incubation, prepared sudan black stain was poured above the LB plate and incubated at room temperature for 30 min and splashed with ethanol. Subsequently, the stained culture plates were again incubated for 30 min and noticed the absorption of sudan black. High absorption is considered as PHB positive. Out of four, only one isolate showed positive for primary screening [29].

2.3 PRODUCTION AND EXTRACTION OF PHB HYDROCHLORIDE - CHLOROFORM EXTRACTION METHOD

The inoculum for PHB production was prepared by transferring pure colonies of isolated bacteria in 50 ml of nutrient broth and incubated for 24 hrs at 37° C. In the following step, grown culture was centrifuged for 15 min at 5000 rpm. Then cell pellets were collected and supernatants were discarded. Then 2 ml of acetone and 2 ml of ethanol were added to remove the dirt. 4% of sodium hypochlorite is added and incubated for 30 minutes in a water bath at 38°C. After incubation 2 ml of chloroform, 1 ml of acetone and 1 ml of ethanol were added to the sample. After that three layers were formed in which the top layer is sodium hypochlorite ,middle layer is chloroform and the bottom layer is PHB [29].

2.4 PREPARATION OF NANOSPHERE IN DIFFERENT SIZE

Nanospheres were prepared using a nanoprecipitation or solvent displacement method with varying polymer-to-drug ratios (4:1, 7:1, and 10:1) as detailed in (Table 2.4). To prepare these nanospheres, 35 mg of the drug was dissolved in 2 mL of water. Simultaneously, different amounts of PHB (140, 245, and 350 mg) were dissolved in 5 mL of acetone. The next step involved slowly adding the drug aqueous solution drop by drop into the PHB organic solution and stirring in magnetic stirring at 500 rpm. Subsequently, this composite mixture was introduced into a 10 mL external aqueous solution while under agitation. This external solution contained 2% (w/v) of poloxamer 407 as a suspension stabilizer. Then the mixture was magnetically stirred at room temperature for 2 h at a speed of 400 rpm to evaporate the organic solvent. The solidified nanoparticles were obtained by centrifugation for 60 minutes at 12000 rpm and 4 °C, then washed three times by resuspending the nanosphere in 5 mL of water and centrifuged to remove any unloaded Drug. Following that, the nanospheres were lyophilized and preserved at 4-8 °C [26]. Further nanospheres were examined by using a Scanning electron microscope.

Formulation	Ratio Between Drug and Polymer	Drug (mg)	Polymer (mg)	Water (mL)	Acetone (mL)	Poloxamer (mL)
E1	4:1	35	140	2	5	10
E2	7:1	35	245	2	5	10
E3	10:1	35	350	2	5	10

Table 2.4. Nanosphere prepared by Nanoprecipitation method

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2.5 PREPARATION OF OINTMENT

After the nanosphere formation of the drug sample. Ointments were prepared in different formulations(O1, O2, O3) of the drug. 0.025g of potassium sorbate is added to the 10 ml of distilled water and then potassium sorbate solution is added to each formulation of drug sample. 0.05ml of paraffin oil is also added to each formulation of the drug sample. Then kept in a magnetic stirrer at 500 rpm until it changes into semi solid consistency[30].

Table 2.5. Ointment preparation

Formultion	Drug	Potassium sorbate (g)	Distilled water (ml)	Paraffin oil (ml)
O1	E1	0.025	10	0.05
O2	E2	0.025	10	0.05
O2	E3	0.025	10	0.05

2.6 ANTIFUNGAL ACTIVITY

Antifungal activity was evaluated with two different fungi. Fungi are penicillium and Aspergillus. Well Diffusion method was used to evaluate with different ointment formulations (O1, O2, O3). The tested strains of penicillium and Aspergillus were inoculated onto the agar plates with cotton swabs. Then, sterile wells containing ointment with each formulation (200 µl) were placed on Rose Bengal Agar and SDA-Sabouraud dextrose agar. The plates were kept at room temperature for two hours to allow the drug to diffuse into the agar; they were incubated for two at room temperature for zone growth. Then inhibition zones were measured and compared further.

III. RESULT AND DISCUSSION

3.1 COLLECTION OF SAMPLE AND ISOLATION OF BACTERIA

In the soil sample, the *Bacillus spp* were isolated and separate colonies were obtained by sequential dilution. In total, four bacterial colonies with various morphological characteristics were selected and the number was assigned to each colony. The colonies were stained on nutrient agar plates and stored for further investigation.

Table 3.1. Colony Morphology

Petri Plate No.	Colony Morphology
B1	Small circular colonies
B2	Large irregular colonies
B3	Medium dried edged colonies
B4	Small circular colonies

Colony Bl is small and circular, which is a typical morphology for *Bacillus spp.* Colonies B2 are large, but they are irregular . This could be due to a number of factors, such as the culture conditions or the presence of other organisms in the sample. Colony B3 is medium and dried, which is another common morphology for *Bacillus spp*. Colony B4 is small and circular.

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3.2 SCREENING OF PHB PRODUCING BACILLUS SPP BY SUDAN BLACK TECHNIQUE:

Table 3.2 PHB Production efficiency

Petri Plate No.	PHB Production efficiency
B1	Maximum absorption of Sudan Black
B2	Minimum absorption of Sudan Black
B3	Minimum absorption of Sudan Black
B4	Moderate absorption of Sudan Black

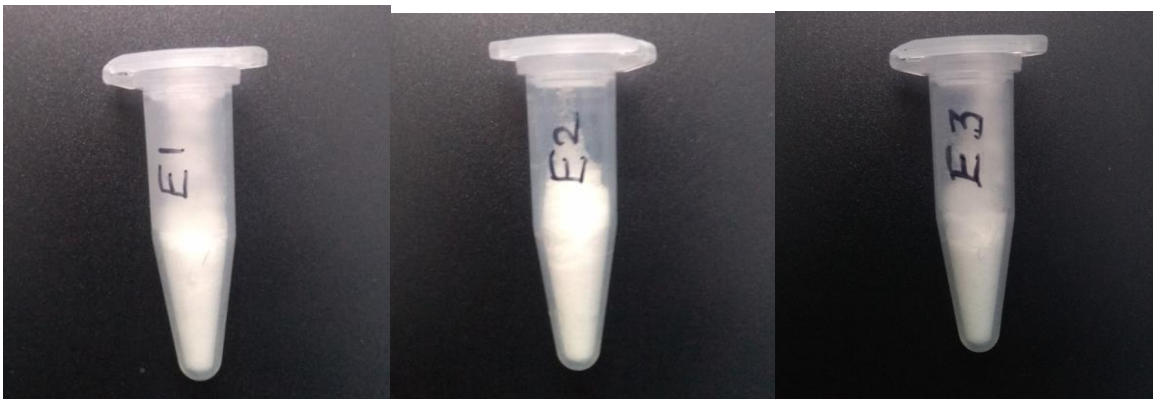
B1 shows the maximum absorption of Sudan Black, which indicates the highest production of PHB. This suggests that the Bacillus sp. in B1 is the most efficient PHB producer. B2 and B3 show the minimum absorption of Sudan Black, which indicates the lowest production of PHB. This suggests that the Bacillus sp. in B2 and B3 are the least efficient PHB producers. B4 shows moderate absorption of Sudan Black, which indicates moderate production of PHB. This suggests that the Bacillus sp. in B4 is a moderately efficient PHB producer. Overall, the table shows that the Bacillus sp. in B1 is the most efficient PHB producer.

3.3 PRODUCTION AND EXTRACTION OF PHB HYDROCHLORIDE - CHLOROFORM EXTRACTION METHOD

The bottom phase (Chloroform with PHB) was precipitated into a Petri Plate and evaporated at a temperature of 70 °C in a Water bath for a period of 20 minutes. The PHB was then collected in an Eppendorf tube.

Figure 3.3 Chloroform extraction

3.4 PREPARATION OF NANOSPHERE IN DIFFERENT SIZE



(a) (b) (c)

Figure 3.4. Nanospheres of (a) Formulation E1 4:1 ratio; (b) Formulation E1 7:1 ratio; (c) Formulation E1 10:1 ratio.

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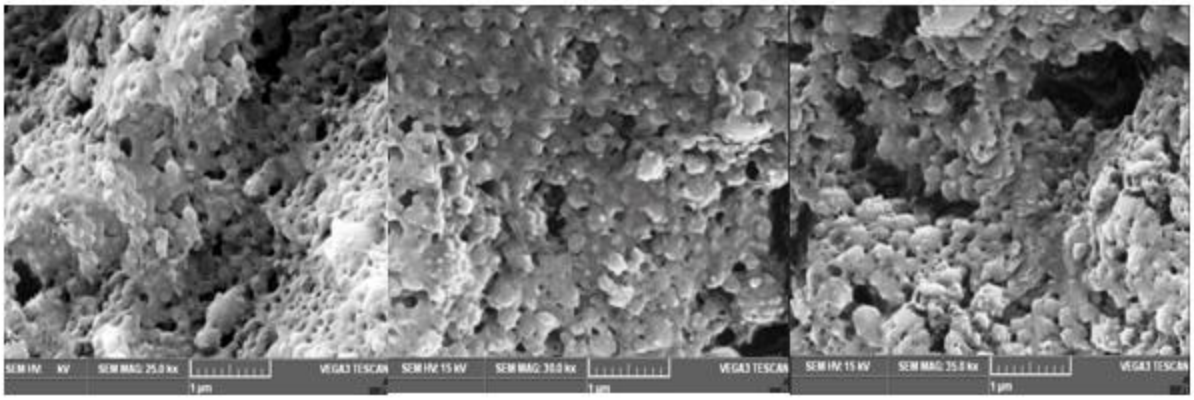
The mean nanosphere size increases as the drug polymer ratio increases. This is because the higher the drug polymer ratio, the more drug molecules are present in the formulation. The average particle diameters were approximately within the range of 124 to 258 nm. This can lead to larger nanospheres being formed. The drug polymer ratio and mean nanosphere size are two important parameters that can be optimized to achieve the desired properties of a nanosphere formulation.



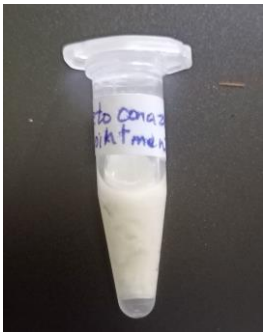
(a) (b) (c)

Figure 3.5. SEM images of (a) Nanospheres E1 4:1 ratio; (b) Nanospheres E2 7:1 ratio; (c) Nanospheres E3 10:1 ratio.

Table 3.3. Nanosphere size of Drug/polymer ratio

Formulation	Drug/polymer ratio	Mean Diameter size (nm)
E1	4:1	124
E2	7:1	146
E3	10;1	258

3.5 PREPARATION OF OINTMENT



Ointments were prepared with different size drug loaded nanospheres.Ointments with smaller nanospheres(O1) can be used to deliver drugs quickly to the skin. This may be useful for treating conditions such as acute pain and inflammation. Ointments with larger nanospheres(O3) can be used to deliver drugs to the deeper layers of the skin. This may be useful for treating conditions such as chronic pain and psoriasis.

3.6 ANTIFUNGAL ACTIVITY

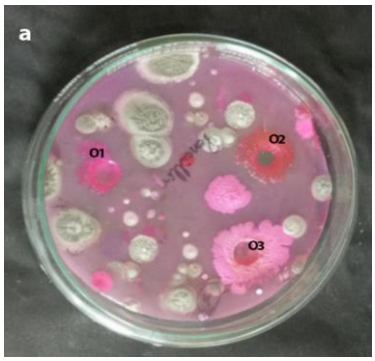


Figure 3.7. Antifungal activity of Penicillium

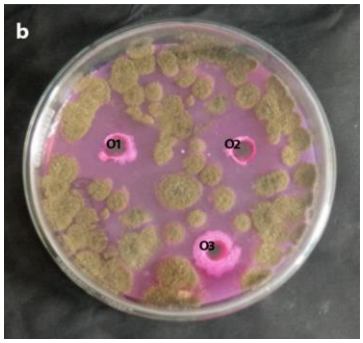


Figure 3.8. Antifungal activity of Aspergillus

Table 3.6. Antifungal activity

Ointment formulation	Penicillium Zone (mm)	Aspergillus Zone (mm)
O1	15	14
O2	12	11
O3	11	10

Results of an antifungal activity of an ointment formulation against Penicillium and Aspergillus by using Rose Bengal Agar plate and SDA-Sabouraud dextrose agar plate. The activity was performed using the well diffusion method. Formulation O1 inhibits the highest zone region while comparing to other formulations.

IV. CONCLUSION

In conclusion, the design and development of different-sized nanospheres for skin application as an ointment containing ketoconazole have presented a promising avenue for enhancing efficacy in dermatological treatments. Through meticulous formulation and optimization processes, we have successfully created nanospheres of varying sizes, each offering unique advantages for skin absorption and therapeutic effectiveness. These nanospheres, when incorporated into the ointment, thereby improving its bioavailability at the target site. The versatility in size selection allows us to tailor the ointment to specific patient needs and skin conditions, optimizing the drug's penetration and retention characteristics.

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