‘Formation of Antifungal Nano Particles of Terbinafine Hydrochloride and its *In-Vitro* Characterization’

MS in Biomedical Sciences

By Rija Irfan

NUST201463162MSMME62414F

Supervisor: DR. NASIR M. AHMED

Co-supervisors: DR. NAVEED AHMED

DR. MUHAMMAD NABEEL ANWAR

Department of Biomedical Engineering & Sciences School of Mechanical & Manufacturing Engineering, SMME Islamabad, Pakistan

September, 2017

National University of Sciences and Technology, NUST Islamabad, Pakistan

'Formation of Antifungal Nano Particles of Terbinafine Hydrochloride and its *In-Vitro* Characterization'

A Thesis Presented to

National University of Sciences and Technology

NUST, Islamabad

In Partial Fulfilment of the requirement for the Degree of

MS in Biomedical Sciences

By

Rija Irfan

NUST201463162MSMME62414F

NUST
NATIONAL UNIVERSITY
OF SCIENCES & TECHNOLOGY

CERTIFICATE OF APPROVAL

Form TH-4

We hereby recommend that the dissertation prepared under our supervision by: **Rija Irfan (NUST201463162MSMME62414F) Titled: 'Formation of Antifungal Nano Particles of Terbinafine Hydrochloride and its *In-Vitro* Characterization' be accepted in partial fulfilment of the requirements for the award of MS degree with grade.**

Examination Committee Members

1. Name: Dr. Umar Ansari Signature: _____
2. Name: Dr. Adeeb Shehzad Signature::_____
3. Name: Dr. Nosheen Signature: _____

Supervisor's name:

Dr. Nasir M. Ahmed Signature: _____

Co-Supervisor's name:

Dr. Naveed Ahmed Signature: _____

Co-Supervisor's name:

Dr. Nabeel M Anwar Signature: _____

_____ _____

Head of Department Date

COUNTERSINGED

_____ _____

Date **Dean/Principal**

DEDICATION

This thesis is dedicated to Allah, to my Parents, to my Supervisors, to my siblings, to my Fiancé and every person who helped me in accomplishing my research work at any step of the way. It is also dedicated to any person who prayed for my success and was a source of support and motivation. Without the above mentioned sources I would not have been able to accomplish this feat.

DECLARATION

I hereby declare that this research study has been done for partial fulfilment of requirements for the degree of Master of Science in Biomedical Sciences. The intellectual content of this thesis is a product of my own work and no portion of the work referred to in this thesis has been submitted in any other degree or other institute of learning. I also certify that the thesis has been written by me. The help I received during my research work and preparation of the thesis, itself has been acknowledged. Moreover, I certify that all sources and literature used have been indicated in the thesis.

Rija Irfan

(NUST201463162MSMME62414F)

CERTIFICATE FOR PLAGIARISM

It is certified that MS Thesis Titled: **'Formation of Antifungal Nano Particles of Terbinafine Hydrochloride and its *In-Vitro* Characterization'** has been examined by us.

We undertake the follows:

a. Thesis has significant new work/knowledge as compared to already published or are under consideration to be published elsewhere. No sentence, equation, diagram, table, paragraph or section has been copied verbatim from previous work unless it is placed under quotation marks and duly referenced.
b. The work presented is original and own work of the author (i.e. there is no plagiarism). No ideas, processes, results or words of others have been presented as Author own work.
c. There is no fabrication of data or results which have been compiled /analyzed.
d. There is no falsification by manipulating research materials, equipment or processes, or changing or omitting data or results such that the research is not accurately represented in the research record.
e. The thesis has been checked using TURNITIN (copy of originality report attached) and found within limits as per HEC plagiarism Policy and instructions issued from time to time.

Signature of Student
Rija Irfan
(NUST201463162MSMME62414F)

Signature of Supervisor
Dr. Nasir M. Ahmad

COPYRIGHT STATEMENT

ACKNOWLEDGEMENT

First of all I would like to praise Allah, the Almighty for providing me with the opportunity to work on my research project and granting me with the ability to successfully bring it to conclusion.

I would like to thank my Supervisor Dr. Nasir for allowing me the opportunity to work on this project and for always being available for his students, for being motivating and very facilitating. I would also like to thank my external Co-supervisor Dr. Naveed from Quaid e Azam University for coming all the way to NUST whenever I required any material and for his constant support and constant supervision throughout the research work. I would also like to thank my Co- Supervisor Dr. Nabeel for being facilitating and supportive.

I am grateful to Dr. Faraz from ASAB for facilitating and overseeing the part of my research work conducted at ASAB. I would also like to acknowledge all the lab engineers and technicians both from NUST and LUMS who helped me in analysis and various parts of my research work.

Lastly, I am thankful for all the help I received from all the seniors from ASAB, SCME and SMME for providing me with the valuable input and teaching me various techniques used for conducting the research.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

HPMC-P	**Hypromellose Phthalate**
DLS	**Dynamic Light Scattering**
FTIR	**Fourier Transform Infra-red Analysis**
PDA	**Potato Dextrose Agar**
FDA	**Food and Drug Administration**
PNP	**Polymeric Nanoparticles**
T-HCL	**Terbinafine Hydrochloride**
PBS	**Phosphate Buffer Saline**
RPM	**Rotation per minute**
PDI	**Polydispersity Index**
UV	**Ultra Violet**

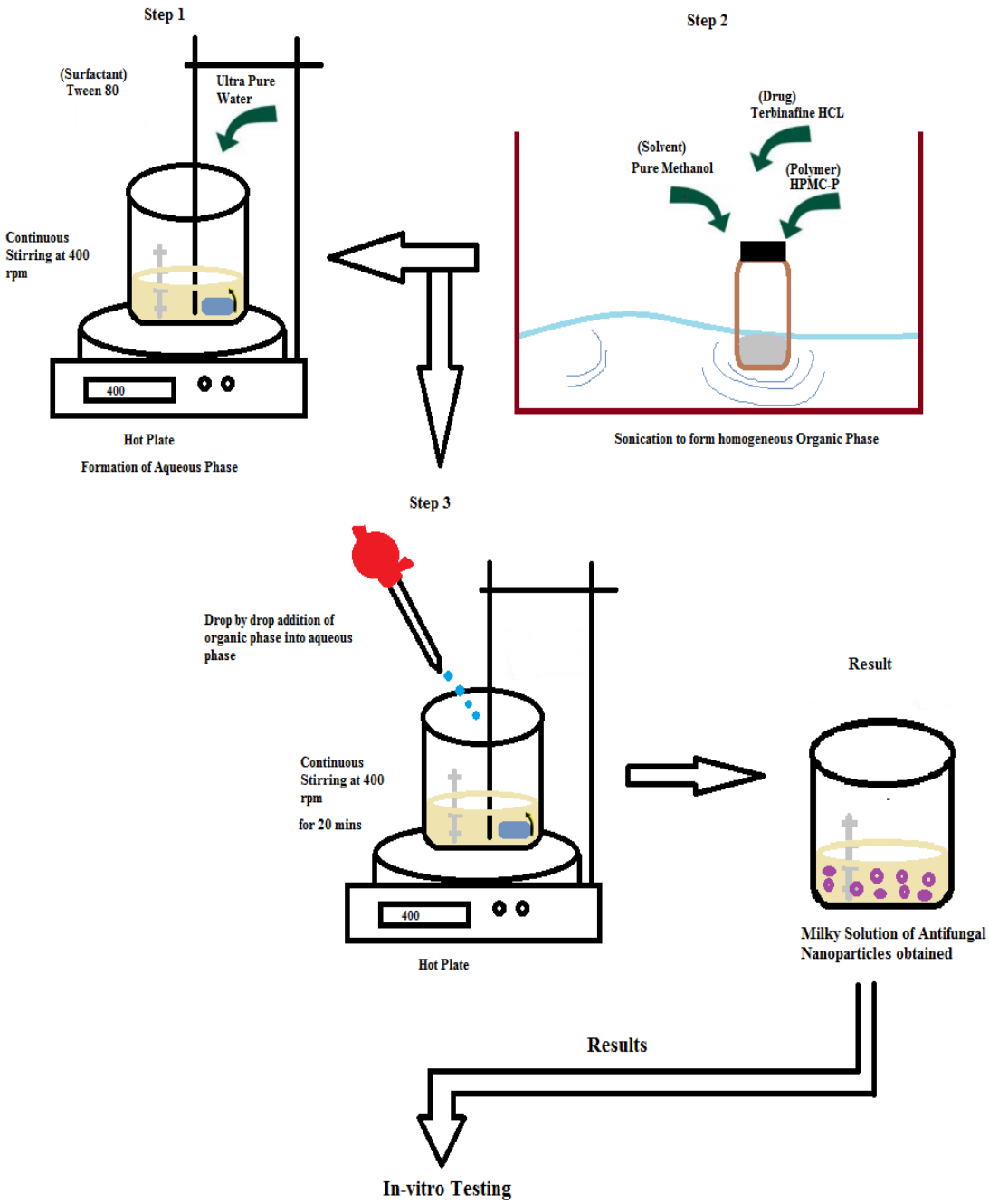
LIST OF TABLES

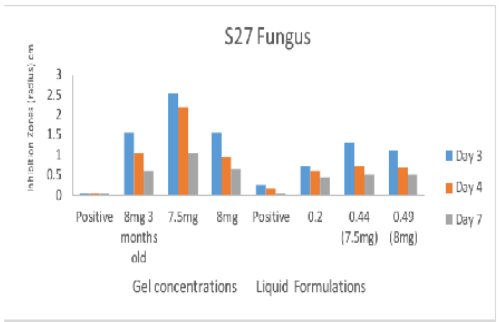
LIST OF FIGURES

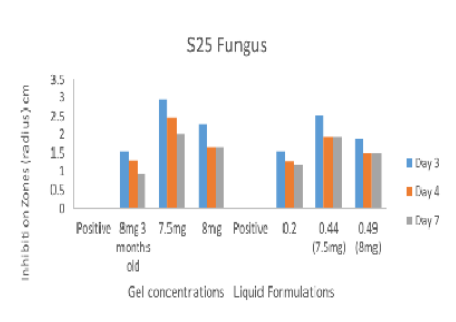
ABSTRACT

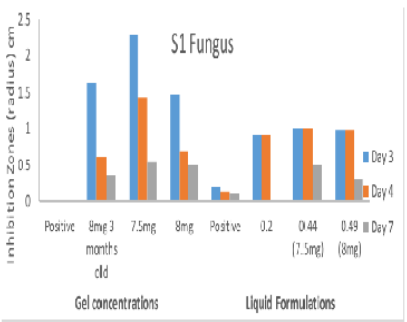
The purpose of this research was to formulate a protocol for development of a low cost, easily manufactured antifungal nanoparticle formulation using HPMC-P as a polymer. Terbinafine Hydrochloride an FDA approved antifungal drug was coated with HPMC-P polymer to form Nano particles which were suspended in a liquid formulation of a homogeneous solution of Tween 80 and Water. Various parameters such as Drug concentration, Polymer concentration, Surfactant Concentration, Organic Solvent Concentration, Temperature of Solution, Stirring Speed and Stirring time were varied to find the most stable and homogeneous drug loaded formulation. Further characterization by Microscopy, Scanning Electron Microscopy, Particle Size Analysis and Zeta Potential, UV Spectrophotometry, Fourier Transform Infra-Red Analysis was performed on the best formulations to find out their morphology, stability, effectiveness and determine the most stable formulation. Further in-vitro drug release studies and in-vitro antifungal assay was performed on the formulations. The liquid formulation was preserved into a Carbopol-937 gel formulation for In-vitro comparative studies in antifungal assay. In-vitro studies showed that Nano-particles showed a more effective response in Gel form as compared to the liquid formulation. The liquid formulation of nanoparticles that showed the best result was milky white in colour, with particle size of 202.9 nm, a poly dispersity index of 0.13, zeta potential of -20, SEM results showing formation of spherical Nanocapsules and FTIR analyses showing bonds found on the surface of the polymer being present on the surface of the nanoparticles confirming that the polymer formed the outer core of the nanoparticles. UV spectrophotometric analysis conducted at 238nm showed an encapsulation efficiency of 85% and in-vitro release studies showed an initial burst of drug after 4h and then a smaller burst after 30h until the release became constant.

GRAPHICAL ABSTRACT









CHAPTER 1

INTRODUCTION

2. INTRODUCTION

2.2 Study Background

According to various studies conducted it is far more difficult to treat a fungal infection or fungal diseases in humans as compared to treat bacterial infections. (AD, 2003) Humans are eukaryotic organisms therefore treating pathogenic fungal infections becomes toxic for the host organism. (Zain, 2011) However, once the surface area to volume ration of antifungal agents is increased more of the agents can be in contact with the fungal pathogens and maximizing antifungal activity. (Nicola Cioffi, 2005; Paulo CS, 2010) Therefore, the action of the antifungal agents can be maximised by formulating them on a micro or nano scale. (Manoj Singh, 2013) Drug delivery has become a very important field of study in medicine and a lot of research is being conducted to form specific and potent drug delivery systems. Polymeric biomaterial provide a lot of benefits as they give widespread chemical diversity. Nanoparticles give the opportunity to further modify these materials as their large surface area has the potential for placement of additional functional groups. Nanoparticles can be used for 'ex-vivo medical diagnostic tests' by manipulating their structures through changes in ph or temperature and interactions with antibodies. (M. Zohri, 2009)

Drug delivery is the delivery of a specific drug to a specific site in the body be it an organ, tissue, cell or physiological site. The system of drug delivery can be made more efficient if the drug is directed right at the target site and not wasted by delivering to unwanted sites. (Yokoyama, 2005)

The rate of drug release depends on a number of factors. Firstly, the solubility of the drug itself, secondly, the desorption/adsorption rate of the drug, thirdly, the diffusion rate of the drug through the nanoparticle core matrix, fourthly, the degradation rate or erosion rate of

the nanoparticle matrix and lastly the combined effect of erosion and diffusion process. (Frankenburg, Glick, Klaus, & Barenholz, 1998)

2.3 Nanoparticles and its importance

The diameter of a Nano encapsulated drug is ranged from 1 to 1000nm in diameter. Nanoparticles are defined as 'solid, submicron-sized drug carriers' which might or might not be biodegradable. Both Nanospheres and Nanocapsules come under the collective term of Nanoparticles. The structure of Nanospheres comprises of a matrix whereas the structure of Nanocapsules comprises of vesicle systems. In Nanospheres the drug is absorbed on the surface or is encapsulate in the sphere. While in Nanovesicles the drug is preserved inside the inner liquid core of the vesicle and is surrounded by polymer which forms a membrane. (M. Zohri, 2009)

The release of drug from Nanospheres occurs by either diffusion or matrix erosion. When the drug is weakly bound in the nanosphere it diffuses faster than the erosion rate of the matrix giving a burst like effect. This in turn shows the method used for incorporation of the drug also has a significant effect on the release of the drug. Secondly, if the drug is coated with a polymer the polymeric layer becomes a barrier for the diffusion of the drug, thus the rate of diffusion of the drug would in turn depend on the solubility of the polymeric membrane. (Ida Idayu Muhamad, 2014)

Nanotechnology presents immense benefit to the pharmacology. Nanoparticles have a large surface area for addition of functional groups on the surface, the large surface area also increases the solubility of the nanoparticles, along with increasing the rate of dissolution of the particles. This in turn makes it efficient as less dosage of drug can give a significant result leading to a fast therapeutic effect. (Vidyavathi Maravajhala, 2012) Nanoparticles

are also reported to protect the drugs from degradation in severe environments such as the gastrointestinal tract. Nanotechnology also leads to the increase in the oral bioavailability of the drug because it shows specialized tools such as 'absorptive endocytosis' this also results in more efficient methods of drug release as the drug is able to release in a stable way and remaining in the blood for longer times which in turn leads to less spontaneous fluctuations and reduced side-effects. The Nanosize of the nanoparticles allows them to enter in to the tissues of the body which allows easy uptake of the particles in the cell. This benefit allows them to target specific tissues or cells without wasting the drug while it dissolute as it travels to its target point. (Pison, Welte, Giersig, & Groneberg, 2006)

2.4 Antifungal Nano Particles

In the years that have passed the number of immunocompromised people has increased which has caused increase in multiple forms of fungal infections. Moreover, there are a limited number of antifungals available and resistance to these antifungal drugs has increased. These downfalls have led to a search for more antifungal agents from various sources. Making these antifungals requires multiple steps and various kinds of methods. (Scorzoni et al., 2016)

Fungi's such as 'Candida Albicans' and 'Trichophyton rubrum' have become resistant to antifungals because of their repeated use against these agents. This calls for research on new antifungal systems as agents such as 'Flucazone' and 'Amphotericin B' are now ineffective against the fungi because of their extensive use. Nanoparticles are therefore the solution to all the above mentioned problems as their multiple benefits allow them to be carrier molecules for these antifungals and they deliver the therapeutic material to the target site of infection maintaining the efficacy of the antifungal drug. (Samrat et al., 2016) Fungicides such as silver nanoparticles have shown to be inhibiting in a broad spectrum of

process in fungi.(Manoj Singh, 2013) Several studies have been conducted on many nanoparticles of zinc oxide, copper and graphene oxide have shown antifungal activity.(Nicola Cioffi, 2005) (He L, 2011; Sawangphruk M, 2012) Charcoals made from wood have also been able to show some antifungal and detoxifying activity as it has a porous structure that results in physical adsorption.(Canilha L, 2004; Ishimaru K, 2001)

2.5 Fungal Skin infections

Surface fungal infections consist of fungal infections of the skin e.g. the ringworm or athlete's foot, the hair, the nails e.g. onchomyoses or the mucous membrane. When it comes to skin infections the most common ones are the dermatophytes, the Candida and The Tinea Versicolor. These infections can be life threatening as well. Species of the Candida, the Cryptococcus neoformans, the Aspergillus species are a serious risk to human life and health. These fungal agents have become very widespread due to the irresponsible as well as larger use of broad-spectrum antibiotics and increase in people who are immunocompromised, as well the usage of venous catheters. (Beck-Sague CM, 1993)

It is reported that around 90% of the fungal infections of the skin are due to 'dermatophytes' which are known as the parasitic fungus that affect the skin, nails, and hair. Terbinafine hydrochloride is now known as one of the top most antifungal drug against these infections. The US FDA has approved of it in cream form, gel form, solution form and spray from. (Anbhule, 2016)

Terbinafine hydrochloride is a relatively new antifungal drug with a lot of potential in fighting antifungal agents. It belongs to the class of antifungals known as allylamine and its action is taken by selective inhibition of the fungal membrane. It is called broad spectrum because it acts against multiple skin infections like yeast, the mold, the fungi and the dermatophytes. (Ozcan et al., 2009)

One of the species of fungal skin infections called Candida are looking for opportunities to infect the membrane of mucus and also the membrane of deep tissues. The drug terbinafine hydrochloride has also shown potential when it comes to fighting against candida, aspergillus and filamentous fungi in in-vitro studies. (Gené J & C, 2001)

2.6 Drug Delivery Systems

Drug delivery systems are known as assemblies of supramolecular which use substances that treat diseases. Nanoparticles have been created to overcome the limitations of traditional drugs such as poor solubility issues, resistance to old drugs, unfavourable pharmokinetics, high toxicity and less uptake of the drug by the target site. Drug targeting is a very important factor in choosing a drug delivery system. Drug targeting is called a mechanism of delivering a specific drug to a specific target area in organs, tissues or cells. The problem with traditional drug delivery systems is that the drug enters the blood stream and is distributed to all the biological sites and not just at the specific target site, this causes the problem of toxicity in the body. Through increasing the delivery to specific target areas and not delivering to unwanted sites we can be able to increase the therapeutic index leading to efficient drug action only at the target site and not at the unwanted areas. (Y, 2005)

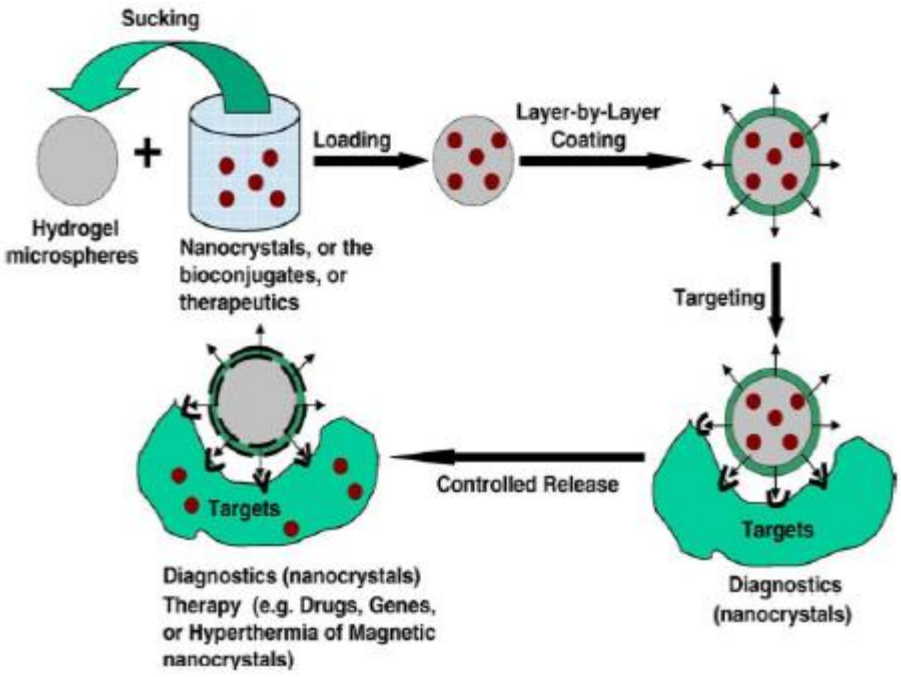


Figure 1: Drug delivery system (Ida Idayu Muhamad, 2014)

2.6.1 Drug delivery system based on Nanoparticles

This is one of the more emerging areas in drug delivery systems as it provides site specific and efficient drug delivery. It has no side effects and also give patient compliance. These systems have several advantages over tradition systems of drug delivery:

1. They are able to surpass the narrowest and smallest capillary routes because of their extra small volume.
2. They can easily enter the gaps between cells and tissues and reach the target organs.
3. They are able to give a controlled drug release which can stay stable for a long period of time. (Ida Idayu Muhamad, 2014)

2.7 Drug Targeting

There are two methods of targeting of drugs, the active method of targeting and the passive method of targeting. Active targeting is the increase in the drug delivery to target sites by using specific unique interactions at the target site whereby applying the drugs pharmacological properties. These interactions involve the following:

1. Antigen and antibody interaction
2. Ligand and receptor interaction

As an alternative some physical signals can also be applied to target sites for active targeting. These signals include magnetic fields and temperature. The carriers of active targeting are the antibodies, thermoresponsive carriers, the transferrin and the ferrite containing liposomes.

Passive targeting on the other hand is a technique in which the physicochemical properties of the carriers lead to an increasing target/non target ratio of the drugs quantity that is delivered. It leads to an adjustment in the histological and physiological properties of the target or nontarget cells, organs or tissues. The carriers for this type of targeting include:

1. Synthetic polymers
2. Natural polymers: the liposomes, the polymeric micelles and the albumin. (Ulrich P., 2006)

Synthetic Polymers	Natural Polymers
1. Poly anhydrides	1. Pectins
2. Poly amides	2. Carrageenan
3. Polyvinyl alcohol	3. Starch
4. Poly lactic acid	4. Cellulose
5. Poly acrylic acid	5. Aliginates
6. Poly cyano acrylates	6. Chitosan
7. Poly isobutylcynoacrylate	7. Xantham Gum
8. Poly ethylene oxide	8. Gellan Gum
9. Poly ortho esters	
10. Poly caprolactone	

Table 1: List of Natural and Synthetic Polymers

2.8 Aim of the Study

The Aim of this study is to create an antifungal treatment to curb the demand for more antifungal medicines as previous antifungal agents are becoming resistant to fungal infections such as Candida because of their repeated use.(Samrat et al., 2016) Further goals for this research are to produce an antifungal formulation that is affordable and easy to form giving an effective result against multiple strains of fungus. For this purpose the drug Terbinafine Hydrochloride was chosen. It is an FDA approved drug (Anbhule, 2016) Terbinafine is marketed under the name of 'Lamisil' in tablets and topical ointments. The problem with the marketed creams is that it needs to be applied for longer periods of time and the oral dosages lead to several side-effects in the body. Side effects for oral dosages may include Diarrhea, Nausea, Liver inflammation, Sensory issues such as reduced sensation of taste etc. For this reason the study required formulation of Terbinafine Hydrochloride Nanoparticles for topical use which are

efficient and can solve the problem of prolong dosages of this antifungal drug. It was planned to make polymeric nanoparticles of the drug using HPMCP as a polymer and Tween 80 as a surfactant for maintaining the structural stability of the nanoparticles and to prevent the nanoparticles from aggregating. (Ravi et al., 2008). HPMCP polymer is used as an enteric coating agent as the drug is has very negligible solubility in water and the polymer has the ability to dissolve in organic solvents (Kim, Park, Cheong, & Kim, 2003) in this case Methanol.

Physical and chemical characterization of the liquid formulation would be done through the techniques of Scanning Electron Microscopy, Particle Size Analysis and Zeta potential, UV Spectrophotometry, and Fourier Transform Infra-Red Analysis. The liquid formulation would be preserved into a Carbopol 937 gel formulation for In-vitro antifungal comparative studies. In-vitro drug release studies will also be conducted on the drug loaded nanoparticle formulation.

CHAPTER 2

LITERATURE REVIEW

3. LITERATURE REVIEW

2.1 Nanoparticles in Medicine and Pharmacology:

The use of Nanotechnology in the field of medicine and pharmacology is on an upward trend. The technology provides immense benefits to scientists in the field of Pharmacy. Scientists are using nanotechnology to study drug delivery and cancer treatment. At a nano scale the surface area of the particles is increased by manifolds, the greater surface area results in greater solubility and also increased dissolving and dissolution rate. Nanoparticles also show increase in bioavailability through oral routes in turn making the use of the drugs very less in dosage. Various nanoparticle based researches have showed how nanoparticles show more stable drug release. The nano scale also helps solve the problem of specific drug targets and the efficacy of the therapy is increased by manifolds. (Vidyavathi Maravajhala, 2012) The small size of the Nano particles provides multiple opportunities for scientist to study the toxicology and curb the side effects that come along with the different drugs. Scientists have also found Nanoparticles to be the pathway to overcome limitations such as the blood brain barrier for delivery of drugs. Moreover, the Nano size also allows for entry into different cellular organs including the nucleus. (Wim H De Jong, 2008)

2.1.1 Nanomedicine:

The term nanomedicine relates to the incorporation of nanotechnology in the field of health care to bring innovation. Particles ranging from 1 to 100nm are the tools for nanomedicine. Nanomedicine requires the benefits of the nanoscale properties of the nanoparticles because they are very different when compared to the same material at a larger scale. (Chang et al., 2015) Nanotechnology's benefits lie in the fact that many mechanisms of the human body also occur at the nano scale therefore targeting these mechanisms becomes easier. Nanoparticles

can easily be used to pass the natural biological passages and membranes, and can easily interact with the biological molecules inside the body whether it be in organs, in tissues or in cells this gives it added benefit of being of use when it comes to gene therapy methods and or methods of delivery of the gene. However, just like all other medicines use of nanomedicines needs to adhere to certain protocol, rules and regulations. It needs to be pertinent to monitoring for the characterization of the nanomedicine, the toxicity of the medicine and most of all the need to pass clinical trials until then its complete potential cannot be determined for use to be beneficial for therapy. That being said, nano medicine has been a big breakthrough in the economical market as it requires less dosages and is efficient thus also making it cost effective which makes it very important for in the healthcare department as it is always looking out for effective and cost efficient medicinal treatments to treat patients for their diseases. (Chang et al., 2015)

2.2 Antifungal Agents

Antifungal agents are described as substances or molecules that lead to suppression in fungal mechanisms of reproduction or growth. In spite of the research on novel molecules and innovative strategies to formulate new molecules of antifungal agents to fight the issue of toxicity and also increase the efficiency of the molecules there still lies a huge demand for discovering new agents of antifungal activity and their characterization. (Agarwal AK, 2008; Martinez-Rossi, 2008; Wiederhold N.P., 2015) A model antifungal agent would have two properties which makes it ideal. It should have a broad range of antigens to work as a broad spectrum agent and it should also show minimal toxicity and side effects. (Carrillo-Muñoz A.J, 2006) A lot of natural sources are a very good resource for finding antifungals. These include resources from plants, animal resources, marine microbe agents or terrestrial agents. (Cruz M.C. & D.S., 2007; Rajeshkumar R, 2012; Rojas JJ, 2006) This is because they have excellent

biosynthetic properties making them a great resource for therapeutic molecules. (Schmidt B, 2008) Recently one fourth of the medicines being used in the world have been derived from natural resources or their products. (Balunas MJ, 2005) One of the most used antifungal drugs 'Amphotericin B' it was discovered from the cultures of the species of Streptomyces nodosus in 1950s. (Trejo WH, 1963) Till the year of 1970 it was thought that fungal infections were easily treatable as a result there was a very low demand for new antifungal medicines. Even before this time there were only two antifungal agents present mainly potassium iodide that was useful for treating sporotrichosis and two types of polyenes called nystatin and amphotericin B. The only little progress in this area was the discovery of flucytosine in the year 1964 till the time the azole drugs were made in the year 1970. Thus, very limited number of antifungal agents are present for the treatment of life taking fungal infections. Moreover, these agents have their limitations for example the nephrotoxicity issue caused by using amphotericin B.(Georgopapadakou NH, 1994) However, recently there is a move towards antifungal research by studying the lipid formulations of the polyenes having less toxic levels and the new types of triazoles that show a broad spectrum action as well as are active against some resistant form of azoles. (F, 2000) Thus, the need for developing new antifungal agents especially occurring from natural resources offering novel pathways of action is an imminent one.

2.2.1 Terbinafine Hydrochloride:

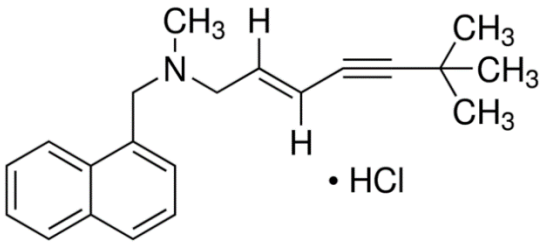


Figure 2: Chemical structure of Terbinafine Hydrochloride (National Centre for Biotechnology Information, 2017)

Terbinafine Hydrochloride a synthetic derivative of the class Allylamine. It is known as a broad spectrum antifungal agent as its range of action comprises of several skin infections e.g the

mold, the fungi and yeast. (Ozcan et al., 2009) Terbinafine Hydrochloride is also very well known to be an active agent when it comes to supressing action of dermatophytes. In fact, according to statistics in various reports almost ninety percent of the skin fungal infections occur due to dermatophytes.(Anbhule, 2016) Terbinafine Hydrochloride is known to be one of the top most antifungal agent used in antifungal agents and has also been approved by the FDA in cream, gel, liquid and tablet forms. (Anbhule, 2016) Terbinafine Hydrochloride is known to be poorly water soluble and has a high affinity for lipids (log P 3.3). It provides the benefit of being used as a topical drug delivery agent as it can be delivered directly on the skin to the affected area, the most major benefit it provides is it only requires low dosage to give an effective result as compared to other antifungal drugs, moreover, it has very minimal toxicity. (Gaba et al., 2015)

Properties of Terbinafine Hydrochloride	
Formula	C21H25N · HCl
Formula Weight	327.89 g/mol
Color	White
Form at room temperature	Powder
Colour at Solubility	Colorless
Turbidity at Solubility	Clear in 50mg/ml of Methanol
Antimicrobial Spectrum	Antifungal, antimycotic and fungicidal against dermatophytes and some yeasts.

Table 2: Properties of Terbinafine Hydrochloride ("Terbinafine Hydrochloride," 2017)

2.2.2 Acting mechanism of Terbinafine Hydrochloride

Terbinafine Hydrochloride is mainly a fungicidal agent against most fungi because of its mechanism of inhibiting 'squalene expidose' in fungi. The fungi that are treated with Terbinafine Hydrochloride have an increased production of squalene which leads to deficiency in the amount of ergosterol production. Ergosterol is an important compound of the fungi's cell membrane, therefore the accumulation of squalene interferes with the development of the fungal cell membrane's function and making of the cell wall. (Ryder, 1992)

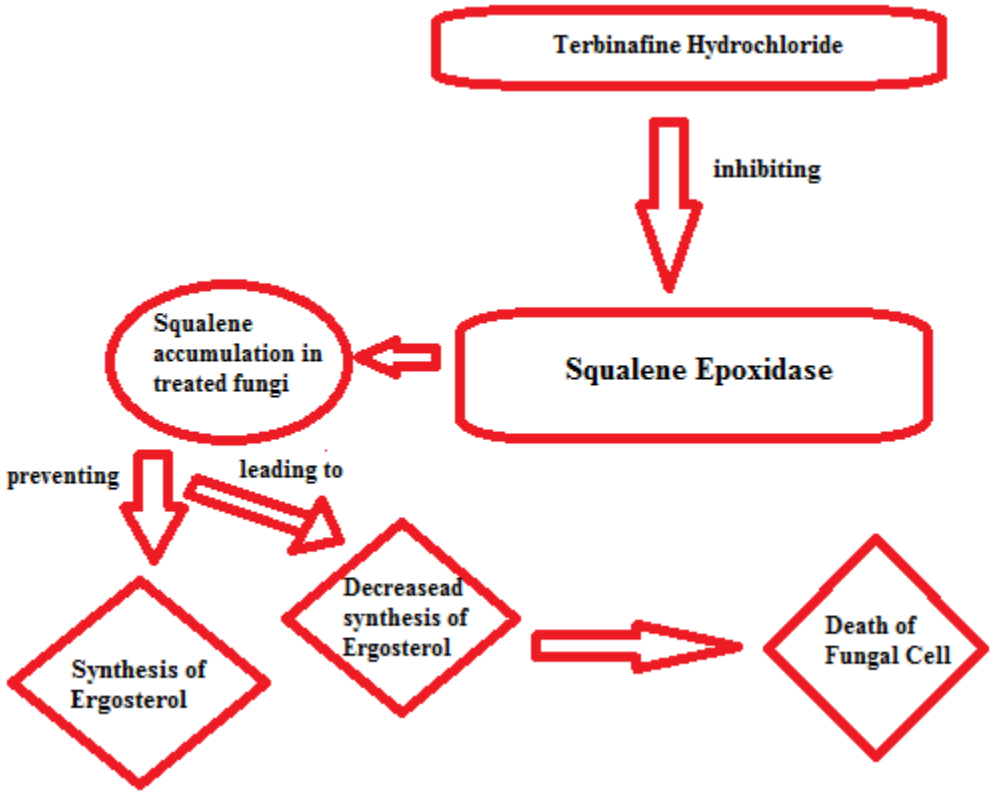


Figure 3: The action profile of Terbinafine Hydrochloride as a fungicide

In fungal infections of the skin Terbinafine Hydrochloride targets the 'stratum corneum' present in the fungal infections. (Alberti, Kalia, Naik, Bonny, & Guy, 2001) Terbinafine Hydrochloride holds the minimum concentration of 0.001- 0.01µg/ml to be used to show

inhibition and also holds a low concentration of 0.003 – 0.006 µg/ml to be used to show a fungicidal effect. (Wavikar & Vavia, 2013)

2.2.3 Previous Work done on Terbinafine Hydrochloride

2.2.3.1 Terbinafine Hydrochloride Chitosan Gels for topical delivery

In this study formulations of terbinafine hydrochloride were made in chitosan gels. The gels were made by using several types of chitosans having a unique molecular weight and were dissolved in diluted form of lactic acid. The complete mixture was made by mixing the materials at an rpm of 600 using a mechanical stirrer. The formulations were then assessed over a period of three months for change in properties and were found to be stable for that time period. The chitosan gels were compared with marketed products and were found to be more easily applicable to the skin when compared to the products found in the market. They also discovered through in-vitro testing that the chitosan gels prepared gave a better result when it came to fighting candida and filamentous fungi species as compared to the market product. Drug loaded chitosan gels also showed better antimicrobial activity against the tested microbes. However, the better activity was also connected with chitosans own antifungal activity. The inhibition zones of the chitosan gels were twice bigger in number as compared to the marketed product therefore making chitosan gels an effective delivery method for topical delivery of terbinafine hydrochloride. (Ozcan et al., 2009)

2.2.3.2 Study on the stable release of Terbinafine Hydrochloride using Acrylamide/maleic acid Gels with sensitivity to pH

The study involved making of polyacrylamide and maleic acid hydrogels using terbinafine hydrochloride as a drug. The study was based on the investigation of drug release. The

hydrogels were made by mixing different quantities of the acrylamide and maleic acid in 1 l of distilled water to make different molar ratios. The gels were then irradiated by placing in polyacrylamide straws and the drug terbinafine hydrochloride was added to the hydrogels by 'direct adsorption' method. The hydrogels were formed by varying the ratio of drug to polymer. Then the effect of the quantity of Maleic acid in the gel and on the capacity of the adsorption of the hydrogel was studied. Secondly the effect of ph on the release of the drug from the gel matrix was also analysed. Results showed that the capacity for adsorption was on an upward trend when the quantity of Maleic acid increased in the gels. The exact quantity of this increase was from 2 to 38mg of the drug per gram of the dry quantity of the gel with increase in Maleic Acid quantity. The reason for this increase was found that increase in Maleic acid led to addition of more acidic groups in the chain and the gel had a higher proportion for swelling. The release studies confirmed that the most imminent parameter of effective drug release is the ph of the solution the gels are added to. Therefore, the gels made through this study have the potential for use in drug delivery systems and can be used for trans dermal application of terbinafine hydrochloride. (Murat S, 2000)

2.2.3.3 Formation of Solid Nano lipid particles of Terbinafine Hydrochloride for Topical Delivery

The study comprised of the formation of 'solid lipid nanoparticles' which could encapsulate drugs of lipohilic nature. A microemulsion of glyceryl monostearate , a glyceral behenate, and Precirol was made and tested on nude mice. They determined stability by analysing particle sizes. The optimal nano particle had a proportion of 5:50 lipid ratio water phase. Moreover, when ethanol was added it had no impact on the penetration of the drug on the epidermis of the mice. In conclusion, when the nano particles were compared with marketed topical drug Lamisil Once it was seen that it might have a better effect for the first 12 hours which could in

turn be a solution for decreasing the longer periods of Lamisil dosage of 24 hours. (Chen et al., 2012)

2.3 The Need to Move from Oral delivery to Topical Delivery of Biomedicines

There are multiple benefits when it comes to switching from oral to the topical use of biomedicines:

1. It helps the wastage of the drug when it undergoes hepatic metabolism.
2. Oral dosages need to be taken with care and cannot be resorted to without the consultancy of a doctor, however, topical medicines are easy to be used without overdosing or affecting internal organs and topical dosages show a better efficacy than oral doses.(Thiruganesh R, 2010)
3. Topical delivery also gives the advantage of being quick in response and having a more stable drug release instead of being dismantled in the GI track as is the case in oral dosages.(Pathan IB, 2009)
4. Topical applications show less danger for application as compared to oral dosages.
5. Oral dosages can have side effects and can also show toxicity inside the body, topical delivery reduces the chance for the drug to be toxic by far. (Alberti I, 2001)

2.4 The Skin as a Passageway for Topical Drug Delivery

The skin has three layers the epidermis, the dermis and the hypodermis. These three layers forms a complex barrier because of the biological organization and the unique chemical composition of all the layers. The epidermis contains ten to fifteern layers of corneocytes giving it a total thickness of ten to twenty micrometer. (M., 2000) The junctions in between cells have lipid envelopes of corneocytes and desmosomes. These structures provide the cells with adhesion with each other. The quality and the crystallinity and the number of the lipids present

in stratum corneum determine what the skin barrier is like or how perfect it is. (Cevc G, 1999) Therefore, the above mentioned structures and the stratum corneum become the major obstacles for the substances that are applied topically on the skin.(Blanco MD, 2003; Essa EA, 2002; J, 2001; Kalia, 2001; Morganti P, 2001; Moser K, 2001; Ting W.W., 2004) There are multiple factors responsible for administering how the drug is released into the skin after application of topical formulations and those are:

1. Molecular weight of the substance
2. The lipohilicity
3. The formulations type
4. Any addition of chemical penetration enhancers
5. State of the stratum corneum physically
6. Level of hydration of the stratum corneum(Morganti P, 2001)
7. Skins metabolic activity

The success of a topical formulation depends on the correlating of the drugs chemical properties and the permeability factor of the stratum corneum. Secondly, the degree of absorption of the drug through the stratum corneum depends on the physical and chemical properties of the drug or the vehicle transporting the drug. Thirdly, the absorption of the drug through diffusion into the stratum corneum to the epidermis and dermis depends on the thickness of the layers, the distance between the layers of the skin, and the capacity of the drug to reach the blood circulation through the cutaneous microvascular system.(Morganti P, 2001)

2.5 Polymeric Nanoparticles

Polymeric nanoparticles are made from biodegradable and a biocompatible polymer with the size ranging between ten to one thousand nano meters. The drug can either be dissolved, it can be entrapped, it can be encapsulated or it can be attached to a matrix of nanoparticle. Thus, whichever method is used for their preparation we can obtain either Nanocapsules or Nanospheres. In Nanocapsules the drug is found in the inner core and is covered with a unique membrane of a specific polymer. Whereas in Nanospheres the drug is uniformly dispersed in the matrix systems. The polymeric nanoparticles are finding use in a broad spectrum of fields ranging from sensors, photonics, medicine, environmental technology, conducting materials, medicine, electronics and pollution control. Polymeric Nanoparticles are very effective vehicles for carrying proteins, drugs or DNA to specific organs or cells. Their small size in nanometres helps to effectively cross cell membrane barriers and can easily travel in the blood stream as well. These nanoparticles can be easily moulded and incorporated in other nanostructure constructs for use in medical applications.(K.E. Geckeler, 1993)

2.5.1 Polymeric Nanoparticles as Drug Carriers

Polymeric Nanoparticles (PNPs) have three mechanisms through which the deliver the drug to the target site:

1. The PNPs swell up through hydration and then the drug is released at the target site through the process of diffusion.
2. The PNPs undergo an enzymatic reaction which makes it rupture, degrade or cleavage at the target size and thus the entrapped drug is released from the inner core.
3. The drug can also dissociate from the polymer through dead sorption and release itself from the swelled nanoparticle.(Yadav H.K.S, 2012)

Biodegradable nanoparticles in literature have been frequently quoted as being used as vehicles for drug delivery because of its bioavailability, efficient encapsulation, a controlled drug release and less toxicity. It also provides the benefit to be utilised as a gene carrier. An amalgamation of polymeric nanoparticles systems with nanostructures gives a very stable drug release. For example hydrogels are used as drug carriers and give a very controlled drug release. (Ida Idayu Muhamad, 2014)

2.5.2 Advantages of Polymeric Nanoparticles in drug delivery

Polymeric nanoparticles have multiple benefits. They are known to be quite stable thus they can be used to incorporate volatile pharmaceutical substances inside its inner core and thus increasing the stability of the substance. Moreover, it is simple to fabricate and is also a cost effective method and can be made in large quantities through multiple techniques. They are an improvement from the old methods of oral and methods of intravenous drug delivery because they are very effective. It provides the capacity to deliver a larger dosage of drug to a target site. The ability to evolve drug release and the choice to choose polymers has made polymeric nanoparticles model candidates for the therapy of cancer, vaccine delivery, contraception and the antibiotics targeted delivery process. Because of their flexible properties polymeric nanoparticles can also be used in other medicinal activities example tissue engineering.

2.5.3 List of Techniques for the Formation of Polymeric Nanoparticle Formulations

Polymeric nanoparticles can be made by two methods either by directly using pre-existing polymers or they can be made from the polymerization of monomers using either classical polymerization or by using polyreactions.(K.E. Geckeler, 1993) Polymeric nanoparticles can be made by many different method. There are a few factors that need to be looked at before the preparation method is chosen and the factors are:

1. The type of Polymeric System
2. The Area of use of the polymeric nano particle
3. The size of the Polymeric Nanoparticle needed for the application

Following is a list of different methods which could be used for preparation of polymeric nanoparticles:

1. Solvent evaporation
2. Dialysis
3. Nanoprecipitation
4. Salting out
5. Supercritical fluid technology (SCF)

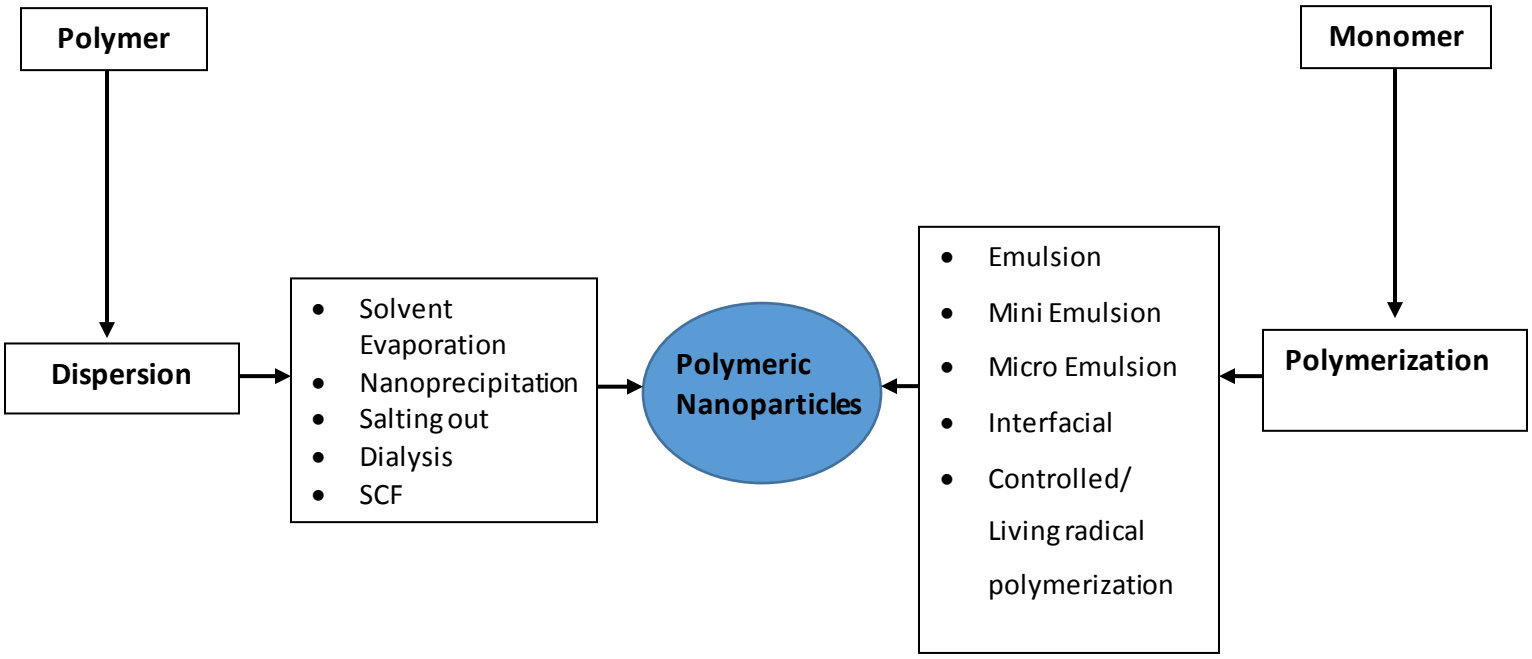


Figure 4: A schematic diagram showing the methods leading to formation of Polymeric Nanoparticles

2.5.3.1 Nanoprecipitation

Nanoprecipitation is the most used method to create formulations for topical administration.(Kim D.G. & J.W, 2006; Lboutounne H, 2004; Shim J., 2004) This method provides many benefits as it's simple and efficient and can be used to easily reproduce

small nanoparticles with a large drug loading range and a very small size distribution. (A. Budhian, 2007; Jiménez M.M., 2004) Nanoprecipitation is a relatively simple technique, requires less energy and resources. It is based on the interfacial deposition because of the displacement of a solvent by a non-solvent. It needs two phases that are miscible in each other:

1. An organic/ oil phase: This phase is made up of a solvent which is organic and is miscible to water for example ethanol, methanol or acetone. The polymer and the hydrophobic drug is added to this phase. Some other substances which may include hydrophobic surfactants, mineral or vegetable oil or triglycerides can also be added to this phase. The oils lead to formation of Nanocapsules instead of Nanospheres. And the surfactant prevents the carrier molecules to aggregate.
2. An aqueous phase: This phase is made up of water but sometimes an addition of hydrophilic surfactants can be made to prevent particles from aggregating. Moreover, aqueous phase can also include polymers to be used as coating substances or if the drug is hydrophilic it can also be added to this phase.

However, for the technique to be applied the solvents need to miscible in each other and we should have a solution of a dilute polymer.

The process of formation of particles in nanoprecipitation techniques comprises of three phases:

1. Nucleation
2. Growth
3. Aggregation (Lince F, 2008)

There are two techniques used for creating polymeric particles through nanoprecipitation:

3. Dialysis: This technique is a competent tool for a controlled and efficient exchange of solvents with water. Targeted solvents usually have high points of boiling. In this

technique solutions of polymer are added into a dialysis membrane and the distilled water surrounding the membrane is replaced several times until the solvent is completely replaced. This removes the molar agents of low mass which could be present in the total solution after polymerization. The nanoparticle suspensions obtained after this are then filtered through a filter paper to remove the aggregates of particles that are larger in size than nanosize.

4. Dropping Technique: The dropping technique is a very simple and easy to use technique that doesn't take much time to conduct. The polymers are dissolved in a hydrophobic solvent and are then added to water drop by drop as the water is being stirred. The hydrophobic solvent is consequently evaporated with time as the solution keeps stirring. This technique also helps form stable nano particles, with a narrow size distribution.(Stephanie Hornig, 2009) The technique is used to encapsulate molecules which are not soluble in water also called hydrophobic molecules but in some cases it has been successful when used with hydrophilic molecules. Many polymers have been used to make nanoparticles by this method. To be used in in vivo methods these polymers need to have biodegradability and biocompatibility. Some polymers could also be coated with new materials to give the polymer surface unique surface properties of adhesion, protection from a certain body system or change its hydrophilicity. (Karim Miladi, 2016)

CHAPTER 3

MATERIALS AND METHODS

4. MATERIALS AND METHODS

4.1. Materials

a) Commercially available drug Terbinafine Hydrochloride obtained from Quaid e Azam University, Islamabad, Pakistan.

b) Ultra-Pure Water used as a solvent for aqueous phase and for making dilutions of emulsions.

c) Hydroxypropyl Methycellulose (HPMC-P) (ordered from MERCK and Co.) was used as polymer.

d) Tween 80 (1.08g/ml) purchased from (ordered from Sigma Aldrich) was used as surfactant.

e) Pure Methanol (ordered from Sigma Aldrich) used as a solvent for organic phase.

f) Carbopol-934 polymer (ordered from Sigma Aldrich) was used as a thickening agent for making Gel.

g) Mono-ethanolamine used as gelling agent in the making of Gel.

h) Commercial grade Ethanol Sigma Aldrich was used for sterilizing equipment's.

i) Sabouraud Dextrose Agar (ordered from MERCK and Co.) media for performing bio assays.

j) Eosin courtesy of ASAB, NUST were used for staining purposes for optical microscopy.

3.2 Fungal Strains

- Fungal strains (S1: Aspergillus Tubingensis and S24: Aspergillus Niger) were obtained from the Atta-ur-Rahman School of Applied Biosciences (ASAB, NUST)

3.3 Apparatus used for Experimentation

a) 50 ml beakers for making aqueous phase.

b) 10 ml vials for making organic phase.

c) 200 ml beakers for making gels.

d) 10ml and 5ml pipettes for measuring liquids.

e) Pipette pumps to be used to pump the liquids up in the pipettes.

f) Magnetic Stirring Bars for stirring the solutions.

g) Glass slides for microscopic observation.

h) Droppers for adding drops of the solutions on glass slides.

i) Double ended spatula for transferring dry chemicals from one apparatus to another.

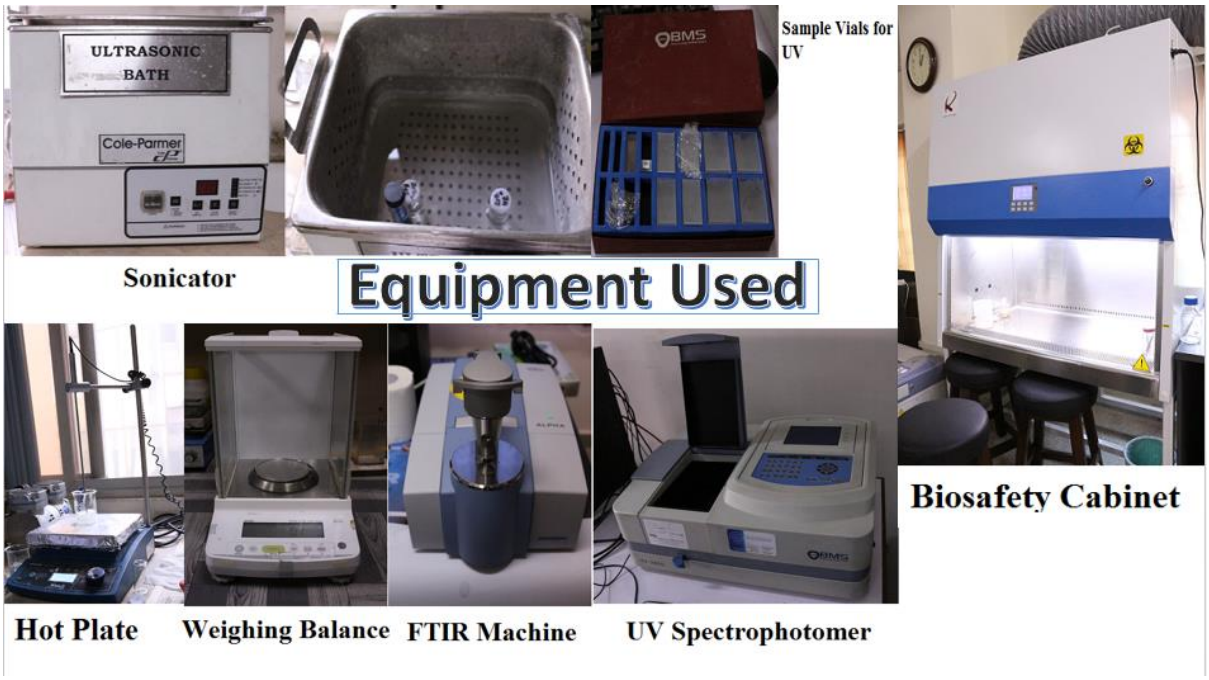


Figure 5: Images of Equipments used in the Study

3.4 Equipment used for Experimentation

a) Hotplate (Wise stir) used for magnetic stirring purposes and maintaining stable temperature.

b) Variable-wavelength UV spectrophotometer (BMS – UV-2800).

c) Vacuum oven (FISTREEN OVA031) and Glass Desiccator were used for drying purposes.

d) Sonicator (Cole Parmer) for proper mixing of organic phase.

e) Centrifuge machine (Siemensstr.25, HERMLE Labortechink GmbH) for obtaining supernatant.

f) Optical light microscope (Amscope) was used for performing microscopy.

g) Ph meter (EcoMet) was used to determine ph of formulations.

h) Electronic Weighing Balance (SHIMADZU) used for measuring dry chemicals.

i) FTIR machine ALPHA Platinum-ATR (BRUKER) was used for conducting FTIR analysis.

j) Analytical Scanning Electron Microscope (JSM- 6490 A) and Ion sputtering Device (Automatic gold coater) (JFC- 1500) was used to conduct SEM analysis of the samples.

3.5 **Methodology**

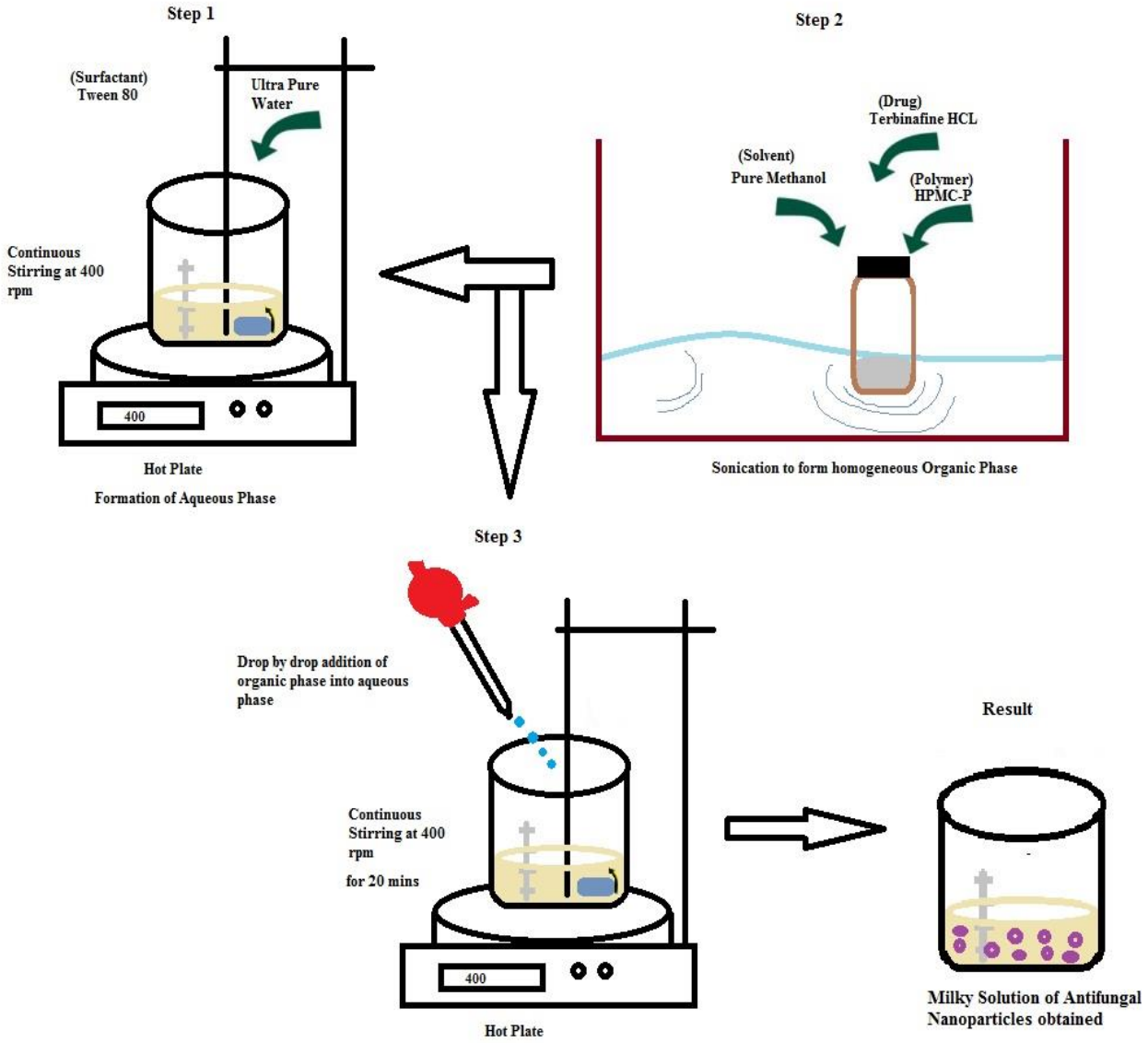


Figure 6: Methodology of Nanoparticle formulation of Terbinafine Hydrochloride

3.5.1 Blank Formulation

To prepare the blank two phases, aqueous and organic were first prepared separately and then the organic phase was mixed into the aqueous phase drop by drop. For the formation of the aqueous phase 10ml of Ultra-Pure Water measured using a pipette was added to a 50ml beaker. The beaker was placed on a hotplate set at 40˚C using a sensor dipped in the water added to the beaker. Then 1.1ml of Tween 80 was measured using a 10ml pipette and added to the water present in the beaker. Both the liquids were then

mixed together on the hot plate using a magnetic stirrer with the stirring speed set at 400rpm until a miscible pale yellow solution was obtained.

To prepare the organic phase 100mg of the polymer HPMC-P was measured on an electronic weighing balance and added to a 10ml vial. 7ml of pure methanol was measured using a pipette and added to the 10ml vial with the HPMC-P polymer added to it. The vial with the solution was then put in a sonicator to mix it properly. The temperature of the sonicator was also maintained at 40˚C. The vial was sonicated until no sign of the HPMC-P particles were seen in the vial and a completely clear solution was obtained.

When both the aqueous and organic phases were obtained the organic phase was taken in a pipette and added drop by drop into the aqueous phase which was being stirred using a magnetic stirrer on the hot plate and the temperature was maintained at 40˚C using a sensor.

The solution was left to be stirred for 15 mins to ensure that all the methanol had evaporated from the solution.

Protocol for Blank Formulation	
Aqueous Phase	
Ultra-Pure Water	10 ml
Tween 80 (Surfactant)	1.1ml
Organic Phase	
Methanol (Solvent)	7ml
HPMC-P (Polymer)	100mg
Drug (T-HCL)	7.5mg
Parameters	
Stirring Time	15mins
Stirring Temperature	40˚C
Stirring Speed	400rpm

Table 3: Protocol for Blank Formulation

Figure 7: Image showing experimental set up on a digital magnetic stirrer

Figure 8: Image showing experimental set up of mixing organic phase in a Sonicator

3.5.2 Drug loaded Formulation

To prepare the drug loaded nanoparticles from the precipitation technique two phases, aqueous and organic were first prepared separately and then the organic phase was mixed into the aqueous phase drop by drop. For the formation of the aqueous phase 10ml of Ultra-Pure Water measured using a pipette was added to a 50ml beaker. The beaker was placed on a hotplate set at 40˚C using a sensor dipped in the water added to the beaker. Then 1.1ml of Tween 80 was measured using a 10ml pipette and added to the water present in the beaker. Both the liquids were then mixed together on the hot plate using a magnetic stirrer with the stirring speed set at 400rpm until a miscible pale yellow solution was obtained.

To prepare the organic phase 100mg of the polymer HPMC-P was measured on an electronic weighing balance and added to a 10ml vial. 7.5mg of the drug Terbinafine HCL was also measured on the electronic weighing balance and added to the 10ml vial with the polymer already added to it. 7ml of pure methanol was measured using a 10ml pipette and added to the 10ml vial with the HPMC-P polymer and Terbinafine HCL drug added to it. The vial with the solution was then put in a sonicator to mix it properly. The temperature of the sonicator was also maintained at 40˚C. The vial was sonicated until no sign of the HPMC-P particles were seen in the vial and a completely clear solution was obtained.

When both the aqueous and organic phases were obtained the organic phase was taken in a pipette and added drop by drop into the aqueous phase which was being stirred using a magnetic stirrer on the hot plate and the temperature was maintained at 40˚C using a temperature sensor dipped in the solution. When the organic phase was completely added to the aqueous phase the solution was left to be stirred for 15 mins to ensure that all the methanol had evaporated from the solution.

Protocol for Drug loaded Formulation	
Aqueous Phase	
Ultra Pure Water	10 ml
Tween 80 (Surfactant)	1.1ml
Organic Phase	
Methanol (Solvent)	7ml
HPMC-P (Polymer)	100mg
Parameters	
Stirring Time	15mins
Stirring Temperature	40˚C
Stirring Speed	400rpm

Table 4: Protocol for Drug loaded Formulation

3.5.3 Drug Loaded Gel Formation

For preparation of the Gel 1g of Carbopol-934 was measured using an electronic weighing balance and added to a 200ml beaker. 100ml of ultra-pure water was measured using a 100ml measuring cylinder and added to 1g of Carbopol-934 added to the 200ml beaker. The solution is then mixed on a hotplate with the solution heated to 50 ˚C and stirred at a speed of 1500rpm to mix the Carbopol-934 in the water. Then the drug loaded nanoparticle formulation is added to the solution as it stirs. Once all the solutions are homogenized, 5ml of Mono-ethanolamine is added to the solution present in the 200ml beaker. The mono-ethanolamine is a gelling agent which solidifies the carpobol-water-nanoparticle formulation mixture into a gel like consistency forming the gel.

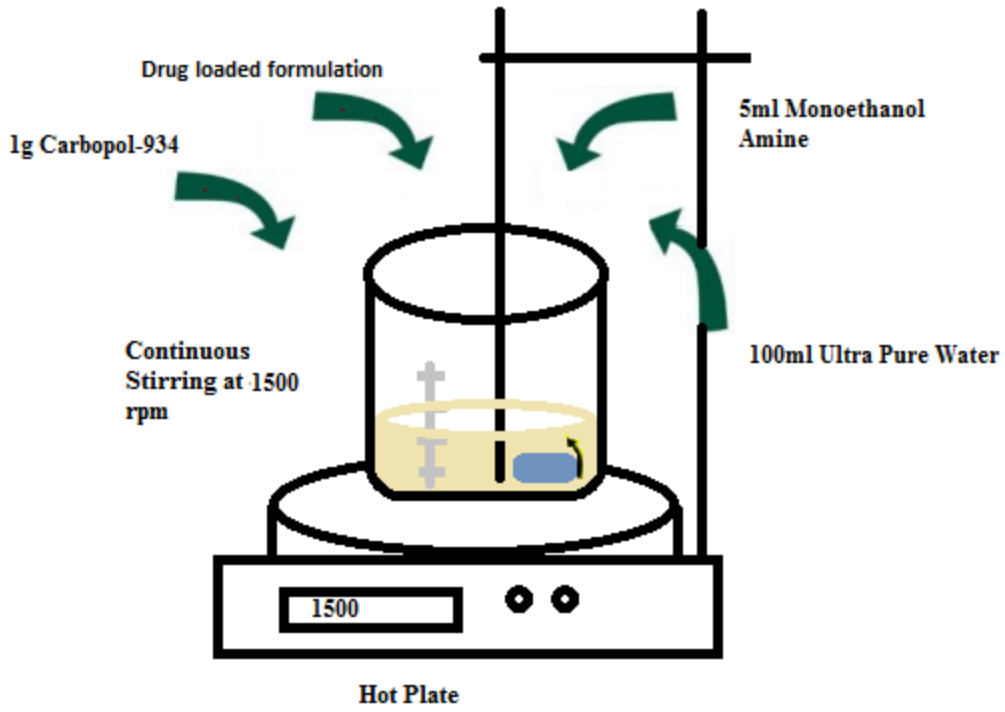


Figure 9: Gel formation of drug loaded sample

3.5.4 Techniques applied for Characterization of Emulsion

3.5.4.1 Stability Studies

The developed formulations were physically observed for three months while they were kept in a dark box at room temperature. The formulations were analysed for precipitate formation and phase separation.

3.5.4.2 Optical Microscopy

For optical microscopy glass slides were prepared of blank and drug loaded samples. An orange pink dye called Eosin received in courtesy from ASAB, NUST was added to the formulations. Then the sample was prepared by taking one drop of each sample in a dropper and dropping one drop of the sample on a glass side for each sample. The glass slides were observed under the optical microscope at the magnifications of 4x, 10x and 50x and screenshots of the observations were taken to see a simple morphology of the molecules.

3.5.4.3 Scanning Electron Microscopy (SEM)

The scanning electron microscopy was performed on the samples to find out the morphology and size of the particles. The emulsion was first diluted by adding one drop of the sample into 5 ml of water taken in a 10ml beaker. The diluted solution is then taken in a dropper and one drop is added onto a glass slide and dried in a desiccator overnight for so as to not damage the morphology before scanning. This is how the sample for conducting SEM is obtained. The samples were then further characterized using the SEM machine to analyse the morphology of the particles. Firstly the sample is put into a vacuum chamber where the ion sputtering device coats the sample with

gold. The SEM machine then forms digital images of various parts of the sample on various magnifications using voltage of 20kV. The images are then analysed to determine the shape of the particles in the sample, the texture of the sample and the size of the particles.

3.5.4.4 Particle Size Analysis (DLS) and Zeta Potential Analysis

For particle analysis the samples were added to 2ml eppendorf tubes and sent to Lahore University of Management and Sciences to conduct the process of Zeta Potential analysis and Particle Size Analysis using a Zeta Sizer machine. Particle size refers to the diameter of the nanoparticles sphere. The zetasizer works on the principle of Dynamic Light Scattering (DLS) determining the Brownian motion of the samples particles to find out the size of the particles in the sample. Particles movement speed depends on their size if the particles are large in size their movement would be slow and if the particles are larger in size their movement will be slow. Thus a zeta sizer takes two pictures after a short time interval of 100μs to see the movement of the particles and thus determines the particle size. Zeta potential uses two measurement techniques to determine the velocity of the particles. It uses the techniques of 'Electro phoresis' and the technique of 'Laser Doppler Velocimetry. Through the combination of these techniques a zeta sizer can measure the fastness of the movement of the particles in a liquid by the application of an electric field through it. Once the speed and electric field is known two other properties of the sample, viscosity and the dielectric constant are used to find out the zeta potential of the given sample.

3.5.4.5 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR machine ALPHA Platinum-ATR (BRUKER) was used for conducting FTIR analysis. The ATR machine allowed direct of the samples in powder or liquid form

without the formation of kbr pellets as required in conventional FTIR machines. FTIR was conducted by taking a drop of the sample and dropping it over the ATR machines scanning point. The head of the ATR is then touched with the sample and the FTIR analysis is conducted and then can be viewed on the monitor using a software specific to the device. FTIR was conducted on blank, drug loaded formulations, the drug in powder form, the polymer in powder form, the surfactant in liquid form and methanol in liquid form.

3.5.4.6 Ultra-Violet spectrophotometry

UV analysis was conducted on both the drug mixed with methanol in which methanol was run as a blank, blank and drug loaded formulations by adding 4ml of the sample in a special UV vial and then the UV machine was run on the sample using a UV software on the computer to find the absorbance spectra of the nanoparticles in the samples. For the blank and drug loaded reading ultra-pure water was run as the blank as the nano particle molecules were suspended in an aqueous solution. The UV spectrum was run within the range of 200-400nm.

3.5.4.7 Entrapment Efficiency and In-vitro Drug Release Study

The Entrapment Efficiency included finding the amount of drug present in the nanoparticles suspended in the sample. For this purpose the blank and drug loaded samples were centrifuged at 13000rpm at 4 degree Celsius for 60 mins. The purpose of this is to separate the nanoparticles from unentrapped particles of drug present in the sample. The pellet of unentrapped particles is obtained and is discarded and the supernatant is then undergone UV spectrophotometry by using distilled water as a

blank. The calibration curve was designed by making dilutions of terbinafine hydrochloride dissolved in methanol. Spectrophotometric analysis was conducted on the aliquots and a graph of absorbance vs concentration was plotted. All readings are observed at a wavelength of 238 nm, the wavelength of the drug Terbinafine Hydrochloride. The encapsulation efficiency is then found by eliminating the amount of the free drug found in the supernatant from the initial amount loaded. (Saadia Ahmed Tayel, 2013) The encapsulation efficiency is then calculated using the following formula:

$$\textit{Encapsulation Efficiency (\%)} = \frac{\textit{(Total drug (initial loaded amount) - Free drug)}}{\textit{Total drug}} \textit{*100}$$

For conducting drug-release study 2ml of the nanoparticle based solution is added to 8ml of phosphate buffer saline (PBS) and added to 15ml eppendorf tubes. The eppendorf are added to an electronic shaker at a speed of 100rpm. Then 2ml of release medium is removed from the solution of PBS for analysis through UV spectrophotometry at wavelength of 238nm and then 2ml of fresh medium is added to maintain the total quantity of 8ml. 2ml of release medium is taken at different time intervals of 15min, 30mins, 1h, 2h, 4h, 6h, 8h, 12h, 24h, 26, 30, 32 and 36h. Drug release studies were replicated twice.

3.5.4.8 In-vitro Antifungal Activity of samples by Plate Diffusion Method

Antifungal testing was conducted by using the plate diffusion method. For this method potato dextrose agar (PDA) was chosen to be made for growth of fungal spores of three different fungus(S1: Aspergillus Tubingensis and S24: Aspergillus Niger) 19.5 Potato Dextrose Agar powder was mixed with 500 ml water to form PDA solution. The

solution is autoclaved and then 20ml of the solution is poured into petri plates. Fungal were spores obtained from Fungal strains present in ASAB, NUST and dissolved in an eppendorf filled with water. Spores are then streaked from the solution on to the PDA. 3 Replicates of each fungal strain for liquid samples and gel samples each were made. Once the fungus are properly streaked on the PDA five wells of the same diameter (5mm) are made in the PDA. The wells are filled with a positive (drug), a negative (blank formulation) and the other three wells are filled with three different ranges of the drug loaded formulation. For the gels four wells are made one with the positive (drug), the other with the negative (blank gel), and two wells with two different drug loaded gel formulations. The antifungal assay is assessed for antifungal activity.

CHAPTER 4

RESULTS AND DISCUSSIONS

4 RESULTS AND DISCUSSIONS

4.1 Optimization of Formulation

To create the optimized formulation the technique of 'Nanoprecipitation' was used in which a hydrophobic drug 'Terbinafine Hydrochloride' was chosen as the antifungal agent and it was planned to encapsulate the drug in a layer of polymer. For this process we needed a hydrophobic polymer that was compatible with the drug as well as an organic solvent in which the hydrophobic drug as well as the polymer could easily dissolve. After reviewing the literature, it was decided to use HPMC-P as the hydrophobic polymer and methanol as an organic solvent. The drug terbinafine hydrochloride is freely soluble in methanol up to fifty milligrams per mili liter. From further tests in the lab it was found that together the chosen drug and polymer freely dissolved in methanol making it the most favourable organic solvent. Other choices were a mixture of ethanol and acetone or pure ethanol but the drug was not found to be as freely soluble in these organic solvents as compared to methanol so from hit and trial and physical observation methanol was chosen as the organic solvent.

For the aqueous phase water was chosen as the aqueous medium and a surfactant had to be added to this medium to ensure the formation of nanoparticles once the organic phase was added to the aqueous phase drop by drop. The surfactant also prevented the nanoparticles from aggregating and thus maintained its shape. From reading the literature it was found that the surfactant tween 80 was soluble in water and formed a miscible solution with water. Tween 80 on its own is quite a viscous pale coloured liquid and it was obtained from Sigma Aldrich. The fact file of Tween 80 obtained from Sigma Aldrich informed the ratio of Water to Tween 80 for forming a miscible liquid was 10:1 therefore it was decided to test this ratio for making the aqueous solution. The quantity of water (10ml) was kept as a constant in the experimentation. Thus, a hit and

trial method was devised to establish the quantities of all the substances needed to prepare the optimized formulation and various parameters were also varied to find the sample that gave the best result.

The quantities and parameters that were varied were as follows:

Parameters Varied													
Drug/mg	4.5	5	5.5	6	6.5	7	7.5	8	8.5	9	10	11	12
Polymer/mg	80	85	90	95	100	105	110	-	-	-	-	-	-
Organic Solvent/ml	4.5	5	5.5	6	6.5	7	7.5	8	8.5	9	9.5	10	-
Surfactant/ml	0.7	0.9	1.1	1.3	1.6	-	-	-	-	-	-	-	-
Stirring Speed/rpm	200	300	400	500	600	800	1500	-	-	-	-	-	
Stirring Time/min	10	15	20	25	30	35	40	45	60	-	-	-	-
Temperature/ °C	20	25	30	35	40	45	50	55	60	-	-	-	-

Table 5: Parameters varied for optimization of nanoparticle loaded formulation

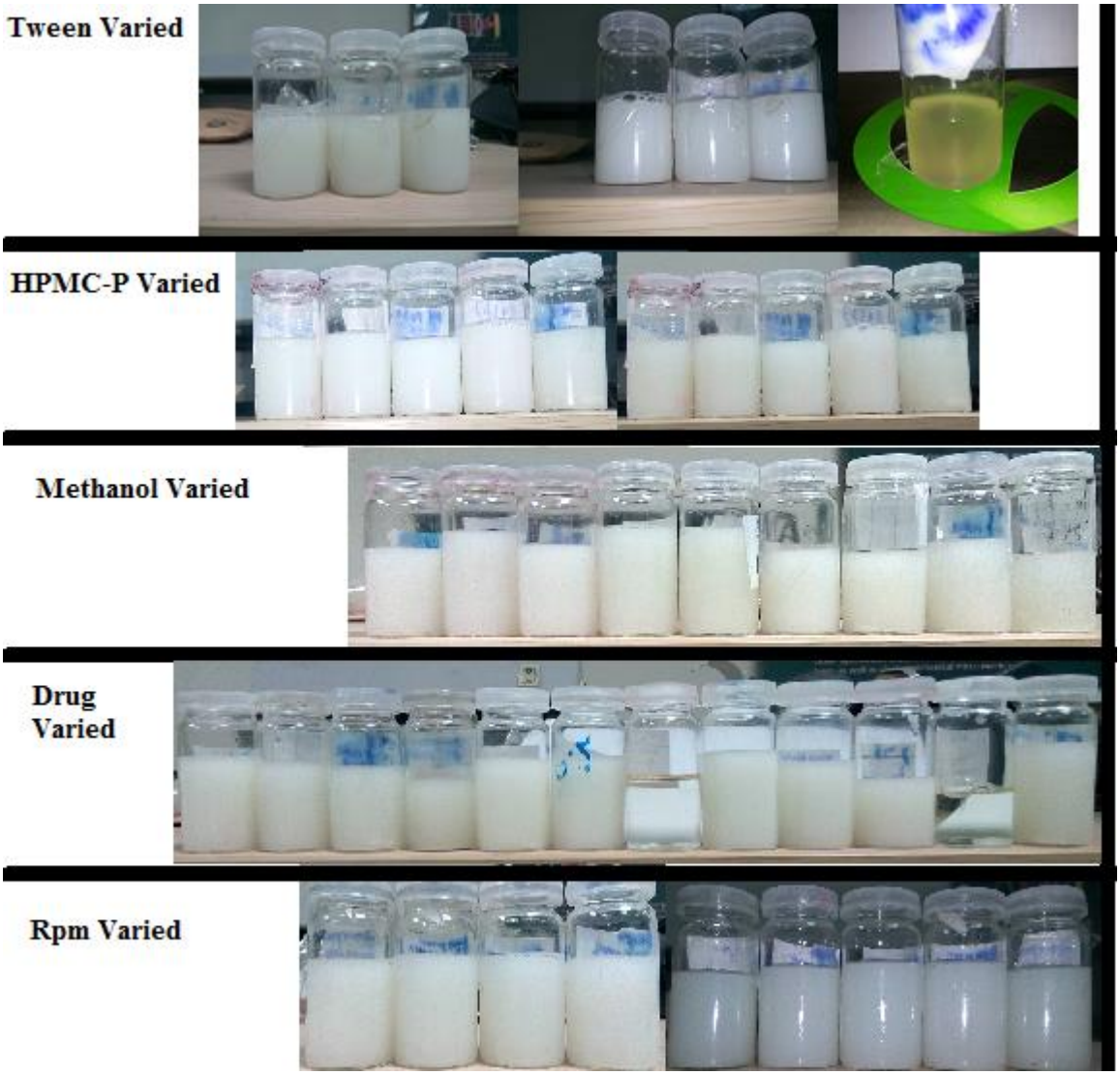


Figure 10: Images of formulations with varied parameters

During the study a few physical factors were made as the deciding factors for rejecting the majority of the formulations so as to shortlist the best ones. These factors were:

1. Homogeneity/ Phase Separation: All solutions that did not show homogeneity once both phases were mixed were rejected. Those that showed phases separation were rejected.
2. Precipitation: Those formulations that had precipitate formations at the bottom after both phases were mixed were rejected.

3. Formation of clumps on the temperature sensor/aggregation within solution: Those formulations that had formed clumps that had stuck to the temperature sensor or that showed big aggregates that had fallen to the ground were rejected.

Therefore, a lot of formulations were made to vary and compare each factor mentioned in the 'parameters varied' table above. Majority of the formulations that were made showed a milky white colour of the liquid formulation. The best formulations that showed little to no precipitation, were homogeneous and showed no clumping on the sensor were shortlisted for further testing and characterization to find the best nanoparticle formulation with small sized particle, high antifungal activity and long term stability. Two formulations were shortlisted based on physical observation of the naked eye and had the following parameters:

Formulation Code	**A**	**B**
Drug/mg	7.5	8
Polymer/mg	100	100
Organic Solvent/ml	7	7
Surfactant/ml	1.1	1.1
Stirring Speed/rpm	400	400
Stirring Time/min	15	15
Temperature/°C	40	40

Table 6: Parameters of Formulation A and Formulation B

These two formulations were then further characterized and formulation A was found to be the best formulation. Each of the formulations were replicated thrice and formulation B showed some precipitation on replication. Whereas, formulation A showed no precipitation on replication, it showed smaller particle size in SEM and also

showed the highest antifungal activity in in-vitro antifungal testing through the plate diffusion method.

4.2 Stability Studies

The chosen formulations were stored for three months at room temperature in a dark shoe box and observed for three months for phase separation or formation of precipitates. All formulations were replicated and seen for any changes in colour or state as mentioned above. No change in colour or phase separation was observed. And no new precipitates than the initial state if any were formed. Formulation A showed the best result staying homogeneous and miscible throughout the time period.

4.3 Optical Microscopy

For optical microscopy Eosin a histological stain that is also hydrophobic was used to stain the nanoparticles and to give a contrast to the images seen in the microscope. All images were observed at 4x, 10x and 50x. A basic idea of the morphology of the particles was obtained from viewing under the light microscope. The particles were observed to be spherical in shape confirming the formation of nano capsules which are made when nano precipitation is used as the method for formulation of nanoparticles.

Images of blank formulation under the light microscope:

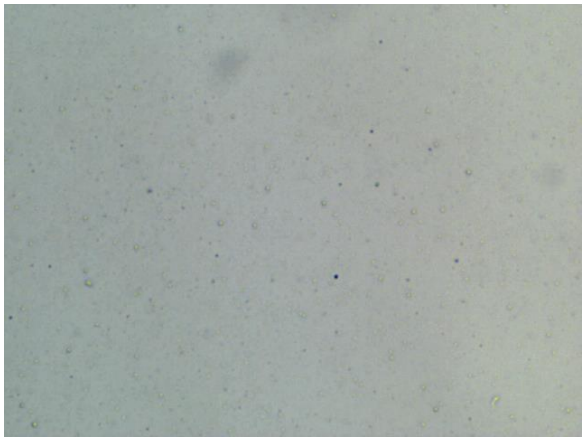


Figure 11: Blank at 4x magnification

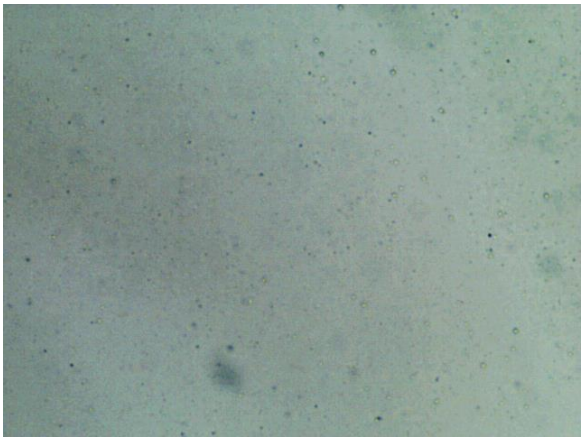


Figure 12: Blank at 10x magnification

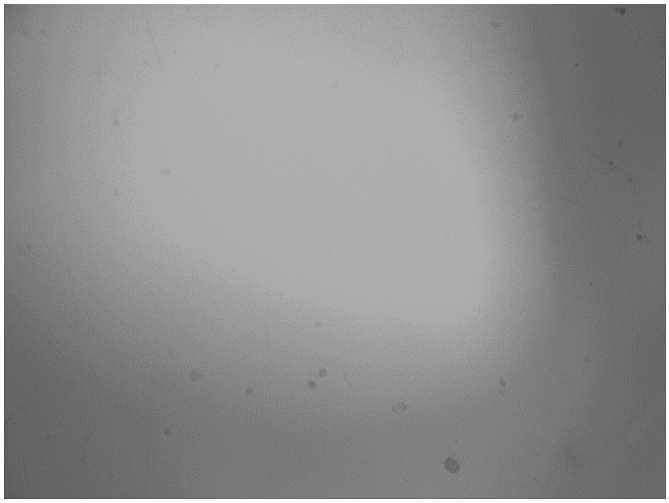


Figure 13: Blank at 50x magnification

Images of Formulation A under the light microscope:

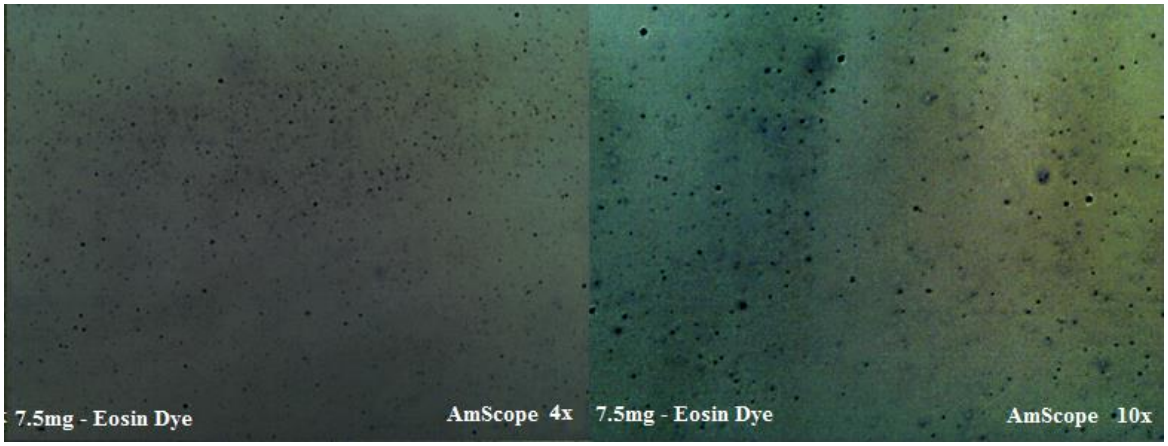


Figure 14: Images of Formulation A in 10x and 4x magnification

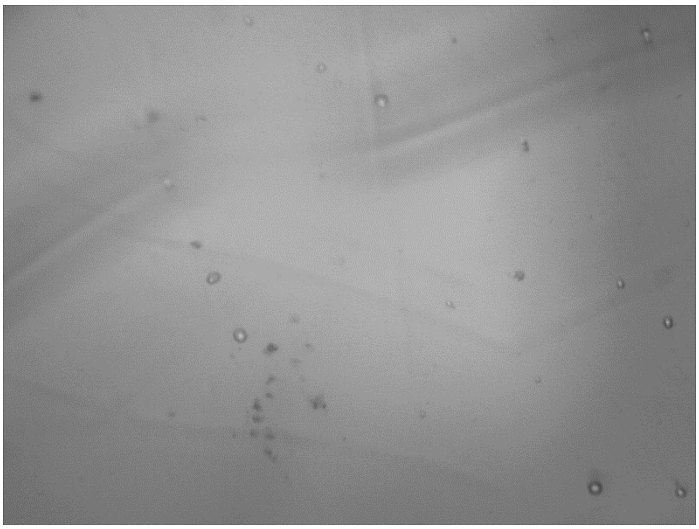


Figure 15: Image of Formulation A at 50x magnification

Images of Formulation B under the light microscope:

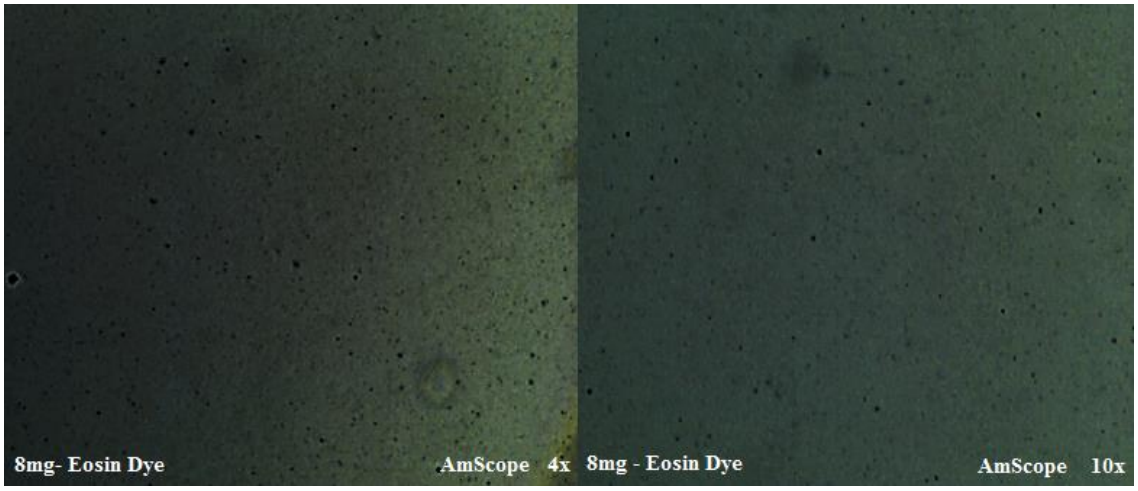


Figure 16: Images of Formulation B in 10x and 4x magnification

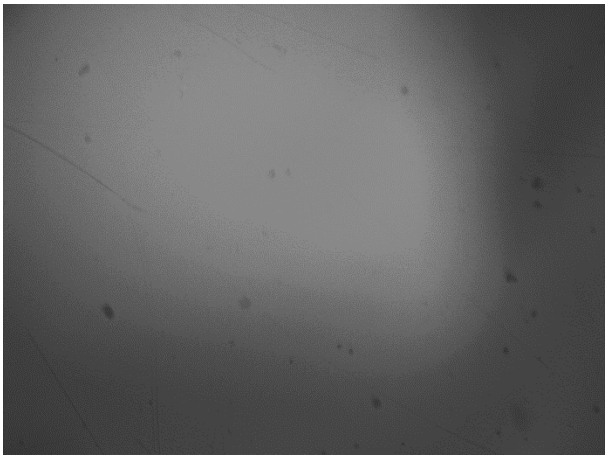


Figure 17: Image of Formulation A at 50x magnification

4.4 Scanning Electron Microscopy (SEM)

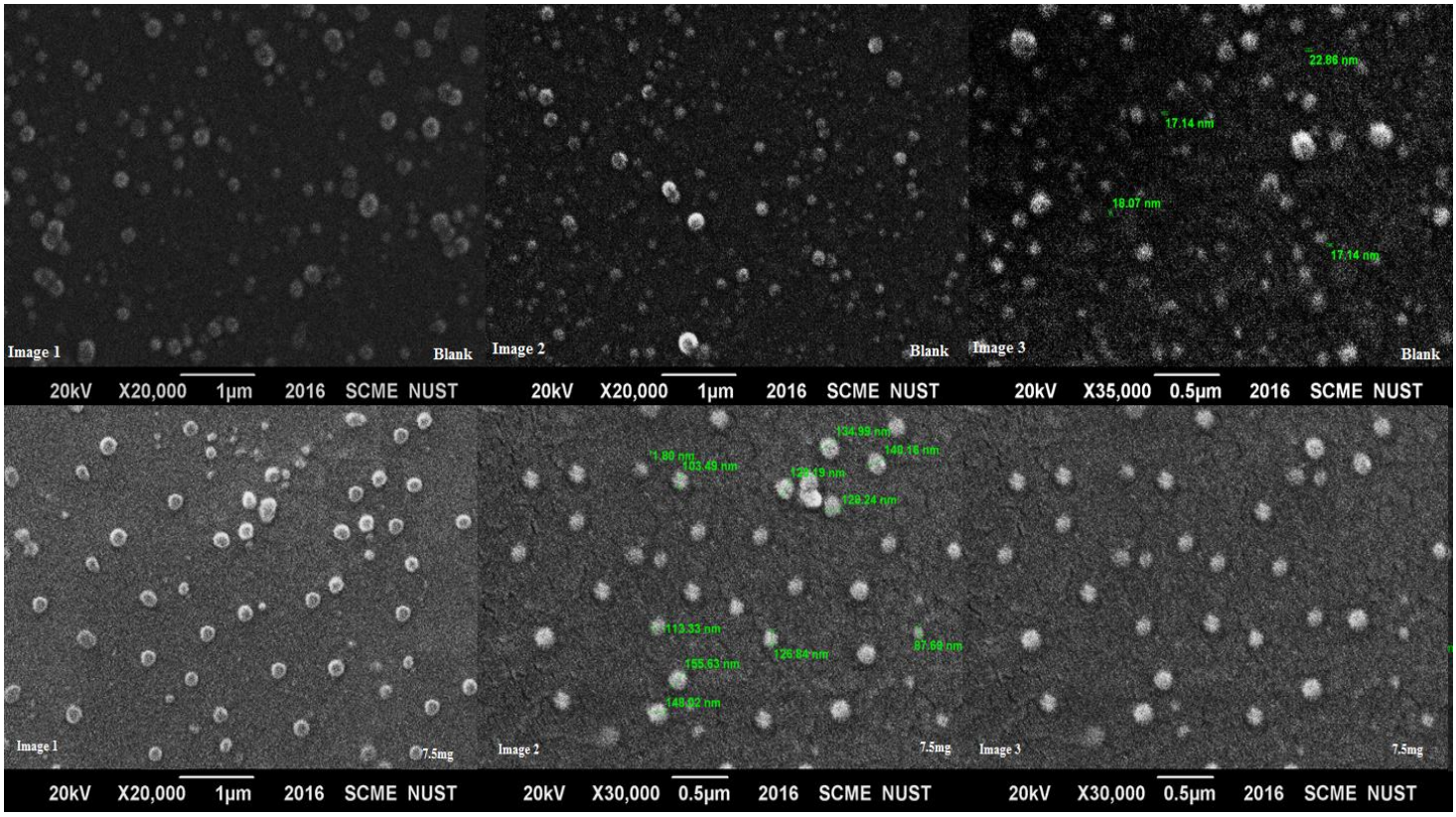


Figure 18: SEM Images of Blank formulations compared with Formulation A

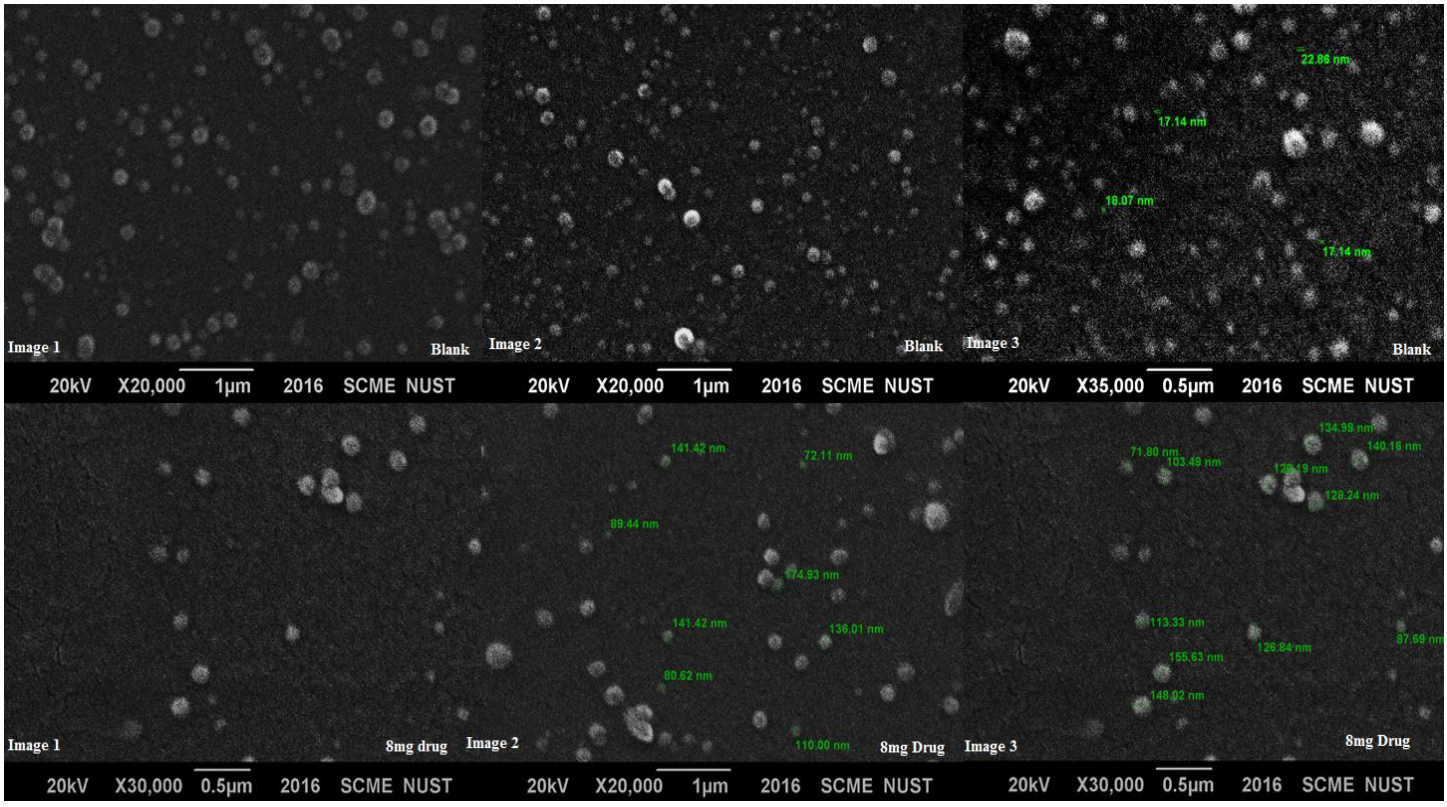


Figure 19: SEM Images of Blank formulations compared with Formulation B

SEM was conducted to observe and analyse the specific external morphology of the formulations. The figures above show the SEM images at a magnification of 20,000x and 30,000x. SEM images of Formulation A and Formulation were compared with the SEM images of the Blank Formulation to see difference in shape and size. The particles in Formulation A once again proved to be better than the particles in Formulation B in size and shape. The particles of Formulation B were less in number and showed disparity in size whereas particles of Formulation A were more uniform and smaller in size than particles of Formulation B. Particles in formulation A were in the mean range of 120nm however, the particles in Formulation B showed a large disparity in size ranging from 89 nm to 160nm. The smaller sized particles were found to be large aggregates of uncombed polymer or empty capsules as they did not match the shape or size of the nanoparticles rendering Formulation A superior and better than Formulation B.

4.5 Particle Size Analysis (DLS) and Zeta Potential Analysis

Formulation	**Hydrodynamic Radius (nm)**	**PDI (Polydispersity index)**	**Zeta potential (mV)**
A	202.9	0.13	-20
B	236	0.2	-21

Table 7: Comparison of Hydrodynamic Radius, PDI and Zeta Potential of Formulation A and Formulation B

Results

		Size (d.nm):	% Intensity:	St Dev (d.n...
Z-Average (d.nm): 202.9	Peak 1:	235.1	100.0	85.40
PdI: 0.139	Peak 2:	0.000	0.0	0.000
Intercept: 0.948	Peak 3:	0.000	0.0	0.000

Result quality : Good

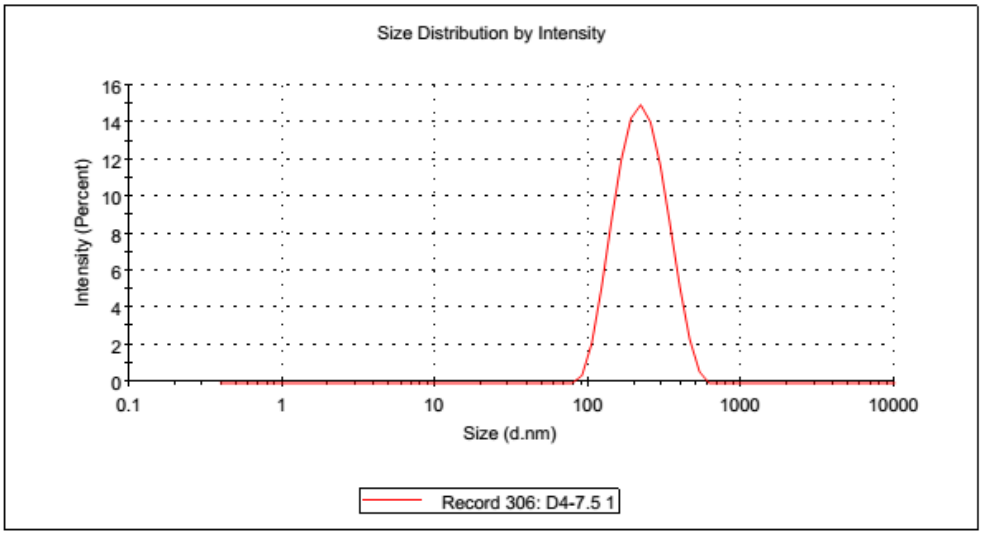


Figure 20: Image of Zetasizer data for size of nanoparticles for Formulation A

Results

		Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -19.9	Peak 1:	-19.9	100.0	5.19
Zeta Deviation (mV): 5.19	Peak 2:	0.00	0.0	0.00
Conductivity (mS/cm): 0.0331	Peak 3:	0.00	0.0	0.00

Result quality : Good

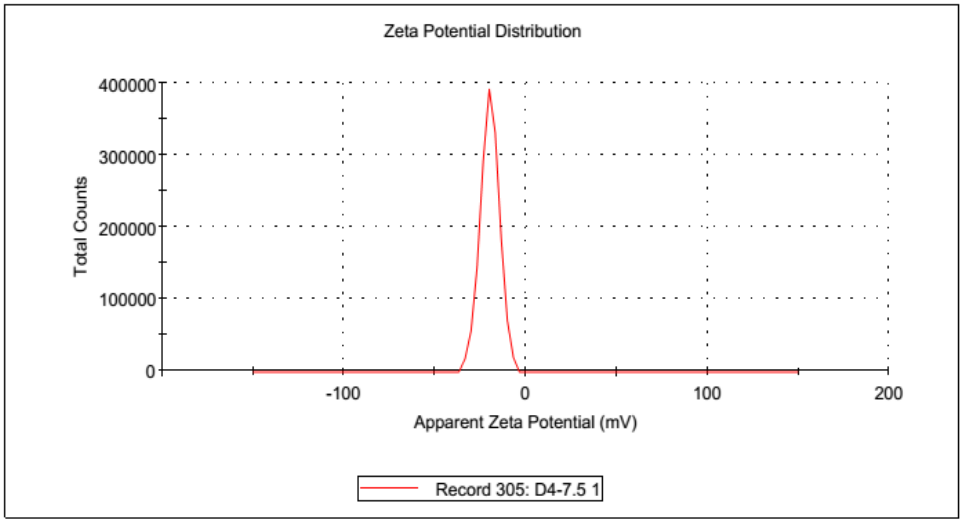


Figure 21: Image of Zetasizer data for Zeta potential of Formulation A

Zeta sizer data showed that the polydispersity index and zeta potential for both the Formulations A and B were good. Both formulations had a PDI less than 0.25 which is a good reading for the poly dispersity index and thus particle sizes were fairly uniform. The Zeta potentials of both formulations were also close to each other showing that the particles also fairly stable. To make the particles even more stable the ph of the formulation could be made more basic as the zeta potential is in negative value. Adding an alkali to the formulation could make the particles more stable. However, the presence of a surfactant in the formulation keeps the nanoparticles stable and prevents any aggregation from happening as seen through stability studies.

4.6 Fourier Transform Infrared Spectroscopy (FTIR)

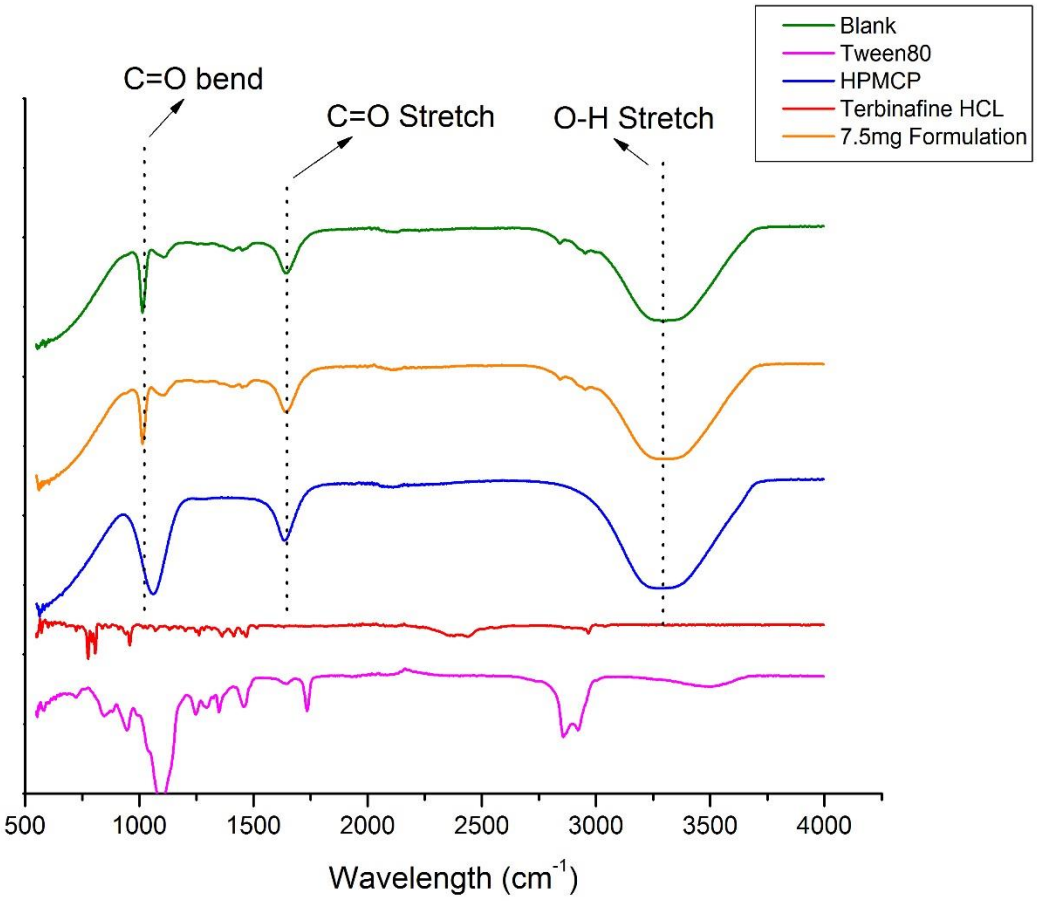


Figure 22: Image showing FTIR of all constituents of Formulation A

All the results of the constituents of the formulation including the blank formulation and drug loaded Formulation A were compiled together on the software 'Origin' to make a comparison and see what bonds were present on the surface of the nanoparticles. FTIR analyses confirmed the presence of C=O bend, C=O stretch and O-H stretch on the surface of the nano particles in both the drug loaded and blank formulations. All these bonds are also present on the surface of the polymer HPMC-P thus proving the nanoparticles were coated with the polymer to make the outer core of the Nano capsule.

4.7 Ultraviolet spectrophotometry, Entrapment Efficiency and Drug Release Study

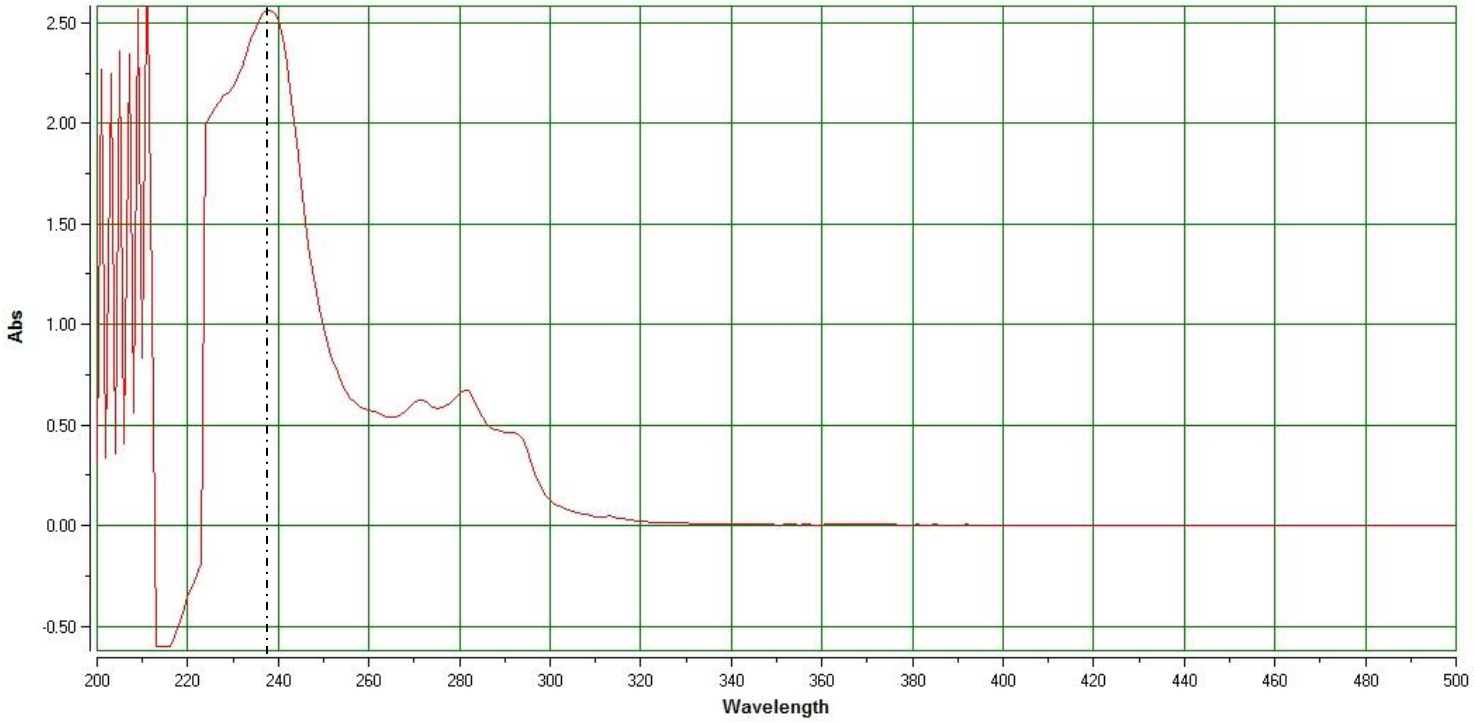


Figure 23: Spectrophotometric analysis of Terbinafine Hydrochloride

The figure above shows the analytical profile of Terbinafine Hydrochloride obtained through the spectrophotometric analysis conducted by the UV spectrophotometer. The highest absorbance of 2.60 is observed at the wavelength of 238nm.

Calibration Curve:

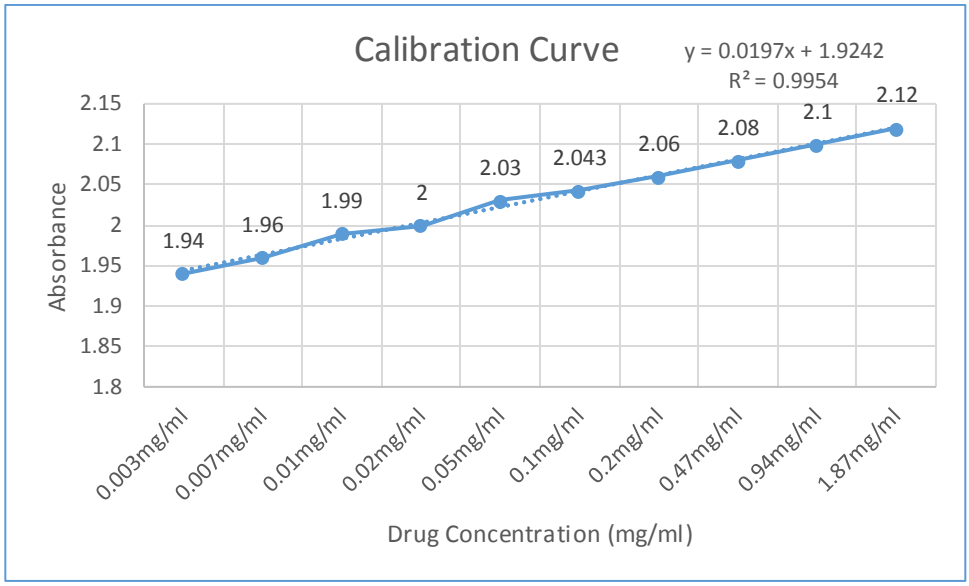


Figure 24: Calibration curve of Terbinafine Hydrochloride

Following are the results of calibration curve of terbinafine hydrochloride by making aliquots:

Concentration	**Absorbance**
0.003mg/ml	1.94
0.007mg/ml	1.96
0.01mg/ml	1.99
0.02mg/ml	2
0.05mg/ml	2.03
0.1mg/ml	2.043
0.2mg/ml	2.06
0.47mg/ml	2.08
0.94mg/ml	2.1
1.87mg/ml	2.12

Table 8: Concentration vs Absorbance values of Terbinafine Hydrochloride Aliquots

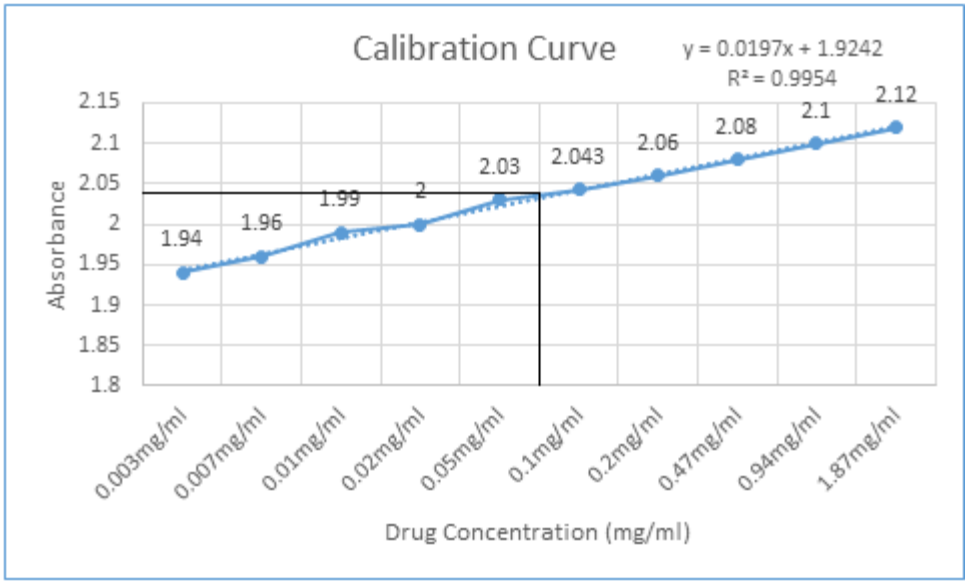


Figure 25: Concentration of free drug in the supernatant

From spectrophotometric analysis of the supernatant it was noted that the absorbance value at the wavelength of 238nm is 2.04. Thus the concentration of free drug in the supernatant was found to 0.1mg/ml. Thus inputting the values in the formula:

$$\textit{Encapsulation Efficiency (\%)} = \frac{\textit{(Total drug (initial loaded amount) - Free drug)}}{\textit{Total drug}} \textit{*100}$$

$$= \frac{0.68\text{mg/ml} - 0.1\text{mg/ml}}{0.68\text{mg/ml}} * 100$$

$$= \mathbf{85.29\%}$$

Therefore encapsulation efficiency was found to be 85.29% which is quite high.

For in-vitro release studies the samples of formulation mixed with PBS solution were taken at various time intervals of 15min, 30mins, 1h, 2h, 4h, 6h, 8h, 12h, 24h, and 36h.

Time (h)	Concentration (mg/ml)
15	0
30	0
1h	0.003
2h	0.0034
4h	0.375
6h	0.1875
8h	0.1
24h	0.02
26h	0.003
28h	0.003
30h	0.168
32h	0.06

Table 9: Time vs drug concentration for in-vitro drug release study

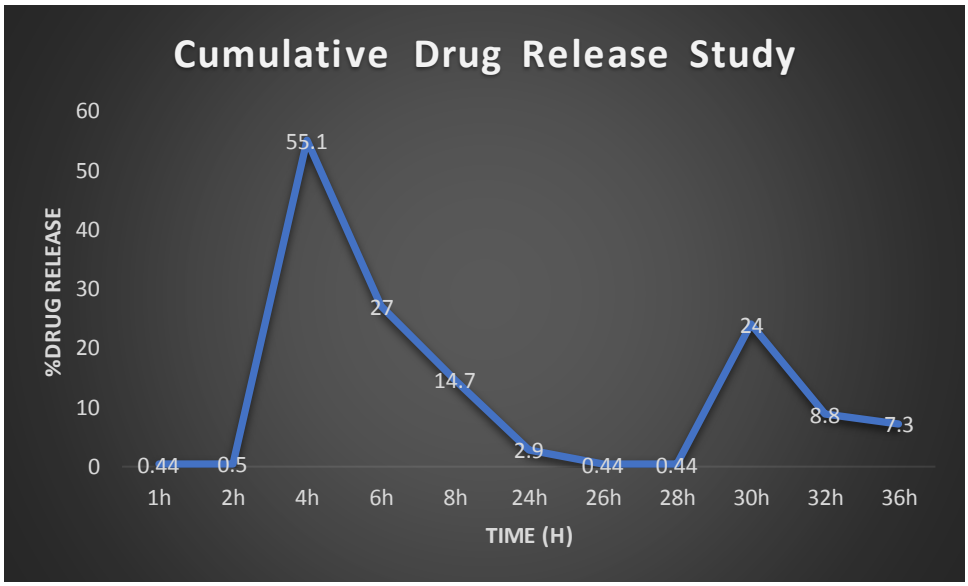


Figure 26: Graph of Cumulative Drug Release

The drug release studies showed the nanoparticles showed little to no drug release for the first two hours. At hour number four there is a large burst of the drug dosage which exponentially declines as the time increases to hour six and eight coming almost to a

minimum after 26 hours. Afterwards, a small burst of drug is again seen at 30 hours which comes to a sharp decline at 32 hours and then almost becoming constant after 36 hours.

4.8 In-Vitro Antifungal Testing by Plate Diffusion Method

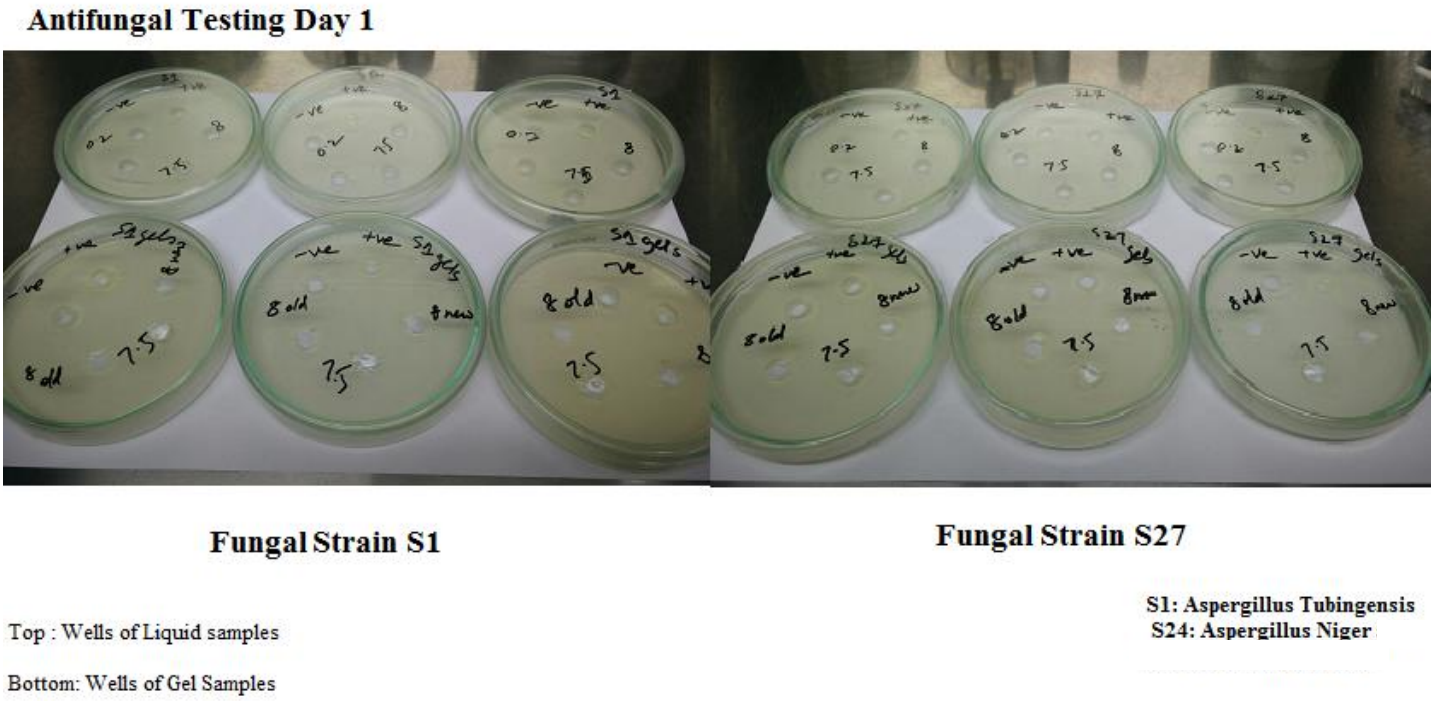


Figure 27: Images of fungal Plates made on day 1

The plates above are as follows:

1. 3 plates of Fungal Strain S1 with 5 wells containing diluted form of Amphotericin B as a positive antifungal drug, blank formulation as the negative, Formulation A (0.68mg/ml/7.5mg of drug), a dilution of Formulation A (0.2mg/ml) and a well of Formulation B (8mg of drug).

2. 3 plates of Fungal Strain S1 with 5 wells containing diluted form of Amphotericin B as a positive antifungal drug, blank gel formulation as the negative, a 3 month old gel formulation, Gel of Formulation A and Gel of Formulation B.

3. 3 plates of Fungal Strain s27 with 5 wells containing diluted form of Amphotericin B as a positive antifungal drug, blank formulation as the negative, Formulation A (0.68mg/ml/7.5mg of drug), a dilution of Formulation A (0.2mg/ml) and a well of Formulation B (8mg of drug).

4. 3 plates of Fungal Strain S27 with 5 wells containing diluted form of Amphotericin B as a positive antifungal drug, blank gel formulation as the negative, a 3 month old gel formulation, Gel of Formulation A and Gel of Formulation B

All the plates were kept in an incubator set at a temperature of 37°C for fungal growth to occur. A lot of precautions were taken to prevent any bacterial growth in the plates:

1. The work place was cleaned with ethanol before putting the petri plates for measurement of fungal growth and antifungal zones on each day.
2. All the plates were made in a laminar flow cabinet using sterilised gloves and once the fungus was plated and the wells were made and filled with the appropriate substances.
3. All the petri plates were properly sealed with a Parafilm to prevent any microorganisms from entering or growing on the agar.
4. The PDA broth was autoclaved before plating it on the Petri dishes.
5. All the petri plates were autoclaved and completely dried before being opened inside the laminar flow for use. Once the petri plates had been plated with PDA it was left to cool down for some time so that the agar could solidify. Once solidified the fungal strains were plated on the agar.

After all plates had been made and sealed they were kept inside a sterile incubator dedicated only for growth of fungi. All the final results were taken once the fungus had fully grown on the agar.

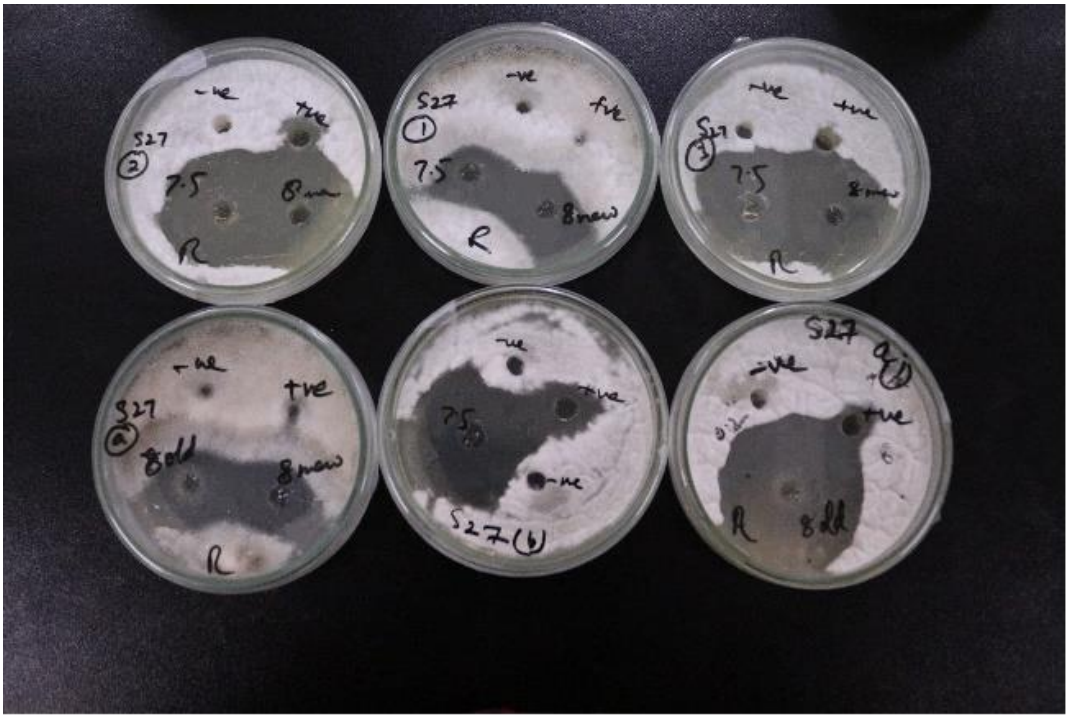


Figure 28: Images of antifungal activity against fungal strain S27 of Gels and Formulations with Terbinafine Hydrochloride as the Positive Antifungal Drug (2ND Replicate)

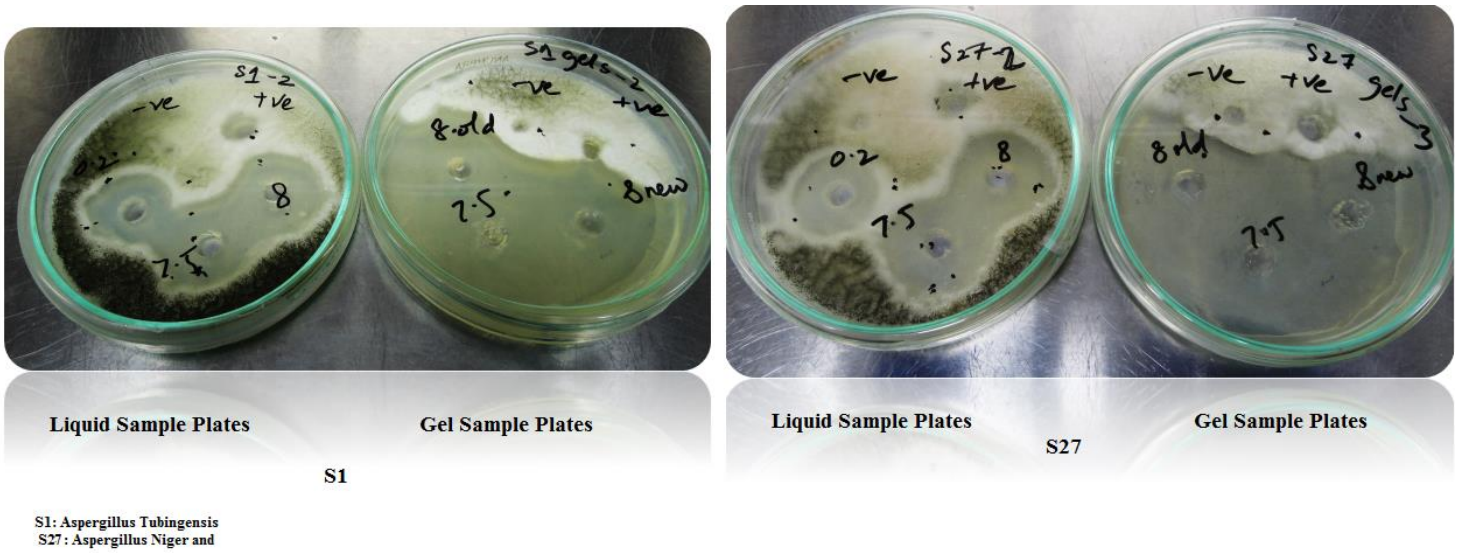
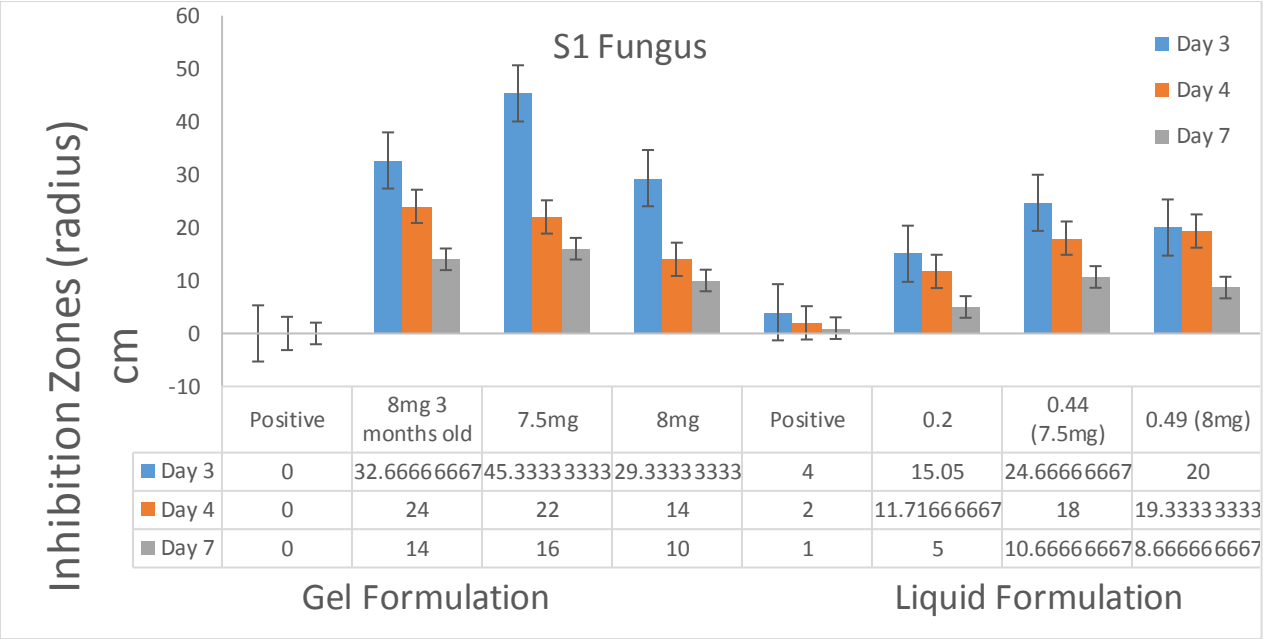


Figure 29: Images of the final results of first antifungal testing on S1 fungus and S27 fungus

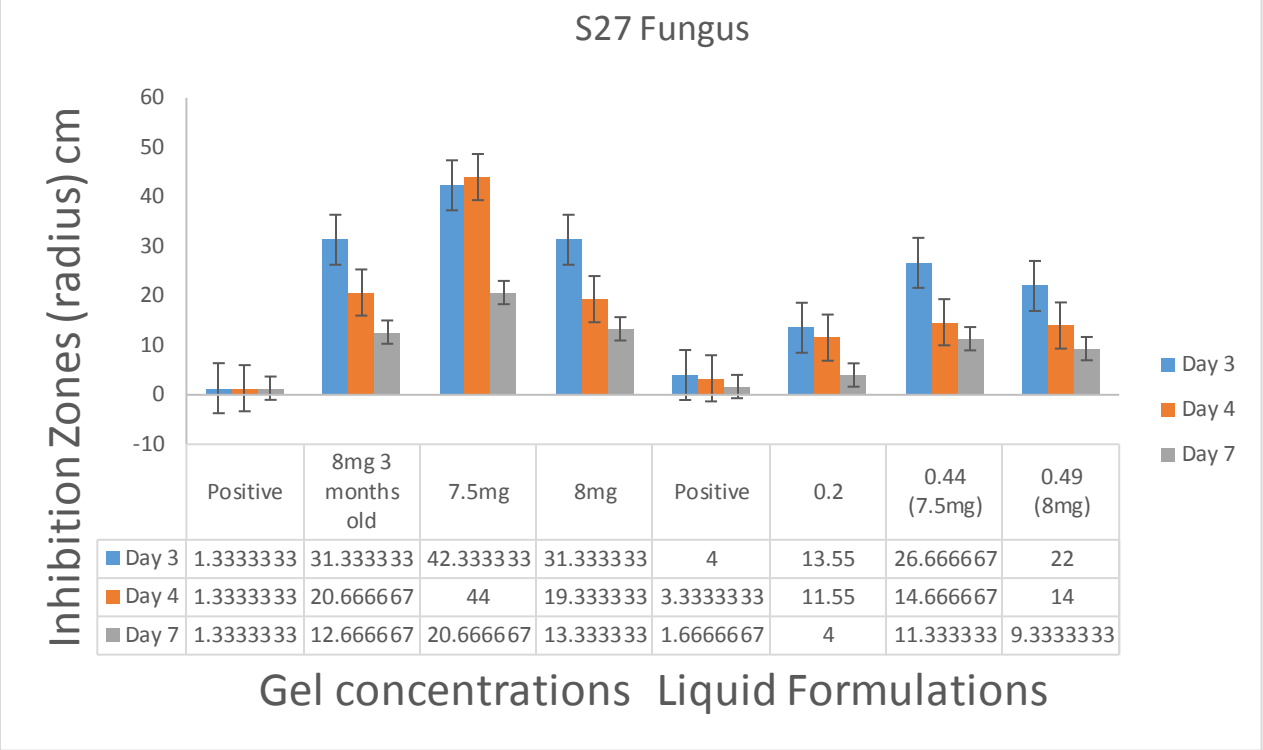
The fungus had fully grown in 7 days and showed proper antifungal zones proving that the liquid as well as the gels showed antifungal capabilities. Upon viewing the results

from the naked eye it can be seen the gels show a greater antifungal activity as they are more viscous and take a longer time to diffuse whereas the liquids diffuse quickly in the plate diffusion method. It is necessary to check the pattern of growth of the fungus to establish that two different fungal strains have grown and no contamination has taken place. In this study it was established that two different strains had grown in each petri plate as the pattern of growth of the fungus, visible from underneath the petri plate was completely different from each other and a uniform pattern was established in each plate.



	Positive	8mg 3 months old	7.5mg	8mg	Positive	0.2	0.44 (7.5mg)	0.49 (8mg)
Day 3	0	32.66666667	45.33333333	29.33333333	4	15.05	24.66666667	20
Day 4	0	24	22	14	2	11.71666667	18	19.33333333
Day 7	0	14	16	10	1	5	10.66666667	8.666666667

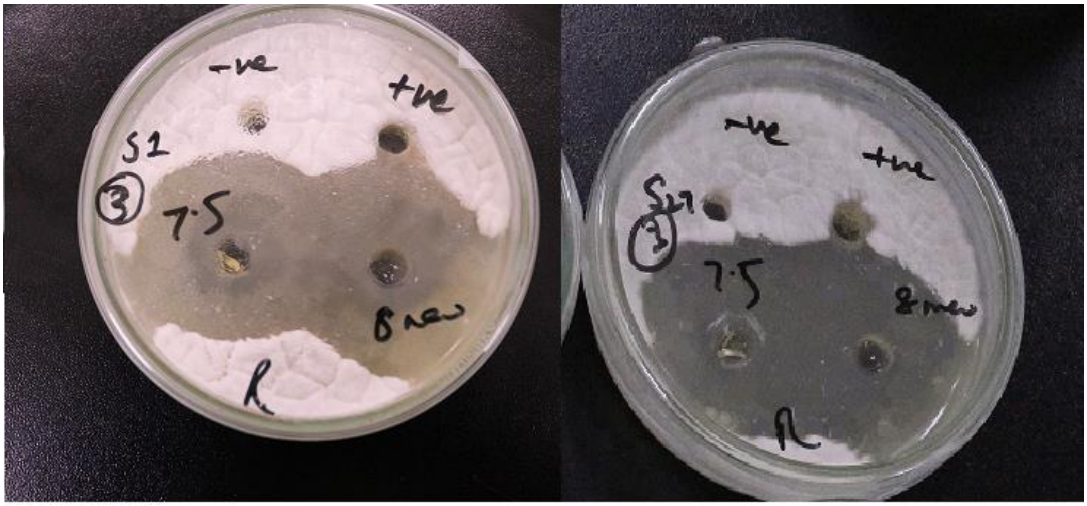
Figure 30: Graphs showing growth of antifungal zones on day 3, 4 and 7 of plating S1 fungus.



	Positive	8mg 3 months old	7.5mg	8mg	Positive	0.2	0.44 (7.5mg)	0.49 (8mg)
Day 3	1.3333333	31.333333	42.333333	31.333333	4	13.55	26.666667	22
Day 4	1.3333333	20.666667	44	19.333333	3.3333333	11.55	14.666667	14
Day 7	1.3333333	12.666667	20.666667	13.333333	1.6666667	4	11.333333	9.3333333

Figure 31: Graphs showing growth of antifungal zones on day 3, 4 and 7 of plating S27 fungus.

The graphs above show growth of fungus over a period of 7 days. The fungus had fully grown by day 7 and did not show further growth. All the petri plates were observed for another week after day 7 but no change in antifungal zones was observed therefore establishing day 7 as the final day for measurements of the zones. From the graphs above it can be seen that the antifungal zones of gels were more than the liquid formulations as gels are more viscous than liquids and thus diffuse slower than liquids. Moreover, comparison between graphs of fungal strain S1 and S27 showed that the antifungal zones of S27 were slightly bigger than the zones of S1 in all the three plates establishing the fact that fungal strain S1 provided more resistance against the formulations and overall it grew faster than S27 over the period of growth. Thus, the formulations showed a better result against S27 when compared with S1 but antifungal activity was observed for both the fungal strains in liquid as well as gel form of the formulations.



S1: Aspergillus Tubingensis
S27: Aspergillus Niger

Figure 32: Images showing antifungal activity of gels of Formulation A and Formulation B

The gel antifungal assay was repeated one more time with the same parameters using only gels of Formulation A and Formulation B as through previous testing it was established that the gels were still showing antifungal activity after three months. Both gels showed large zones of fungal inhibition and were almost comparatively the same.

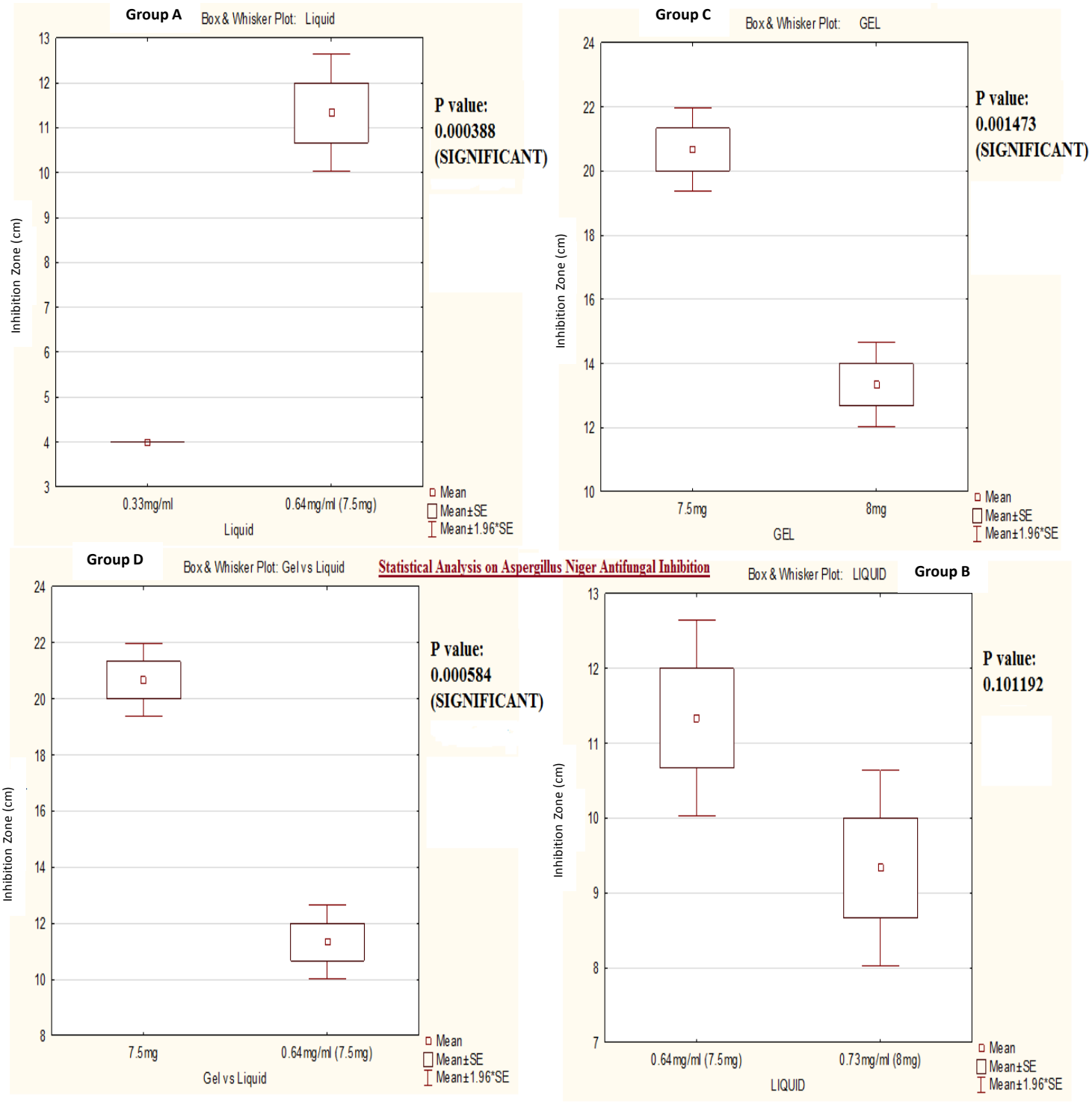


Figure 33: ANOVA test of Fungus Aspergillus Niger

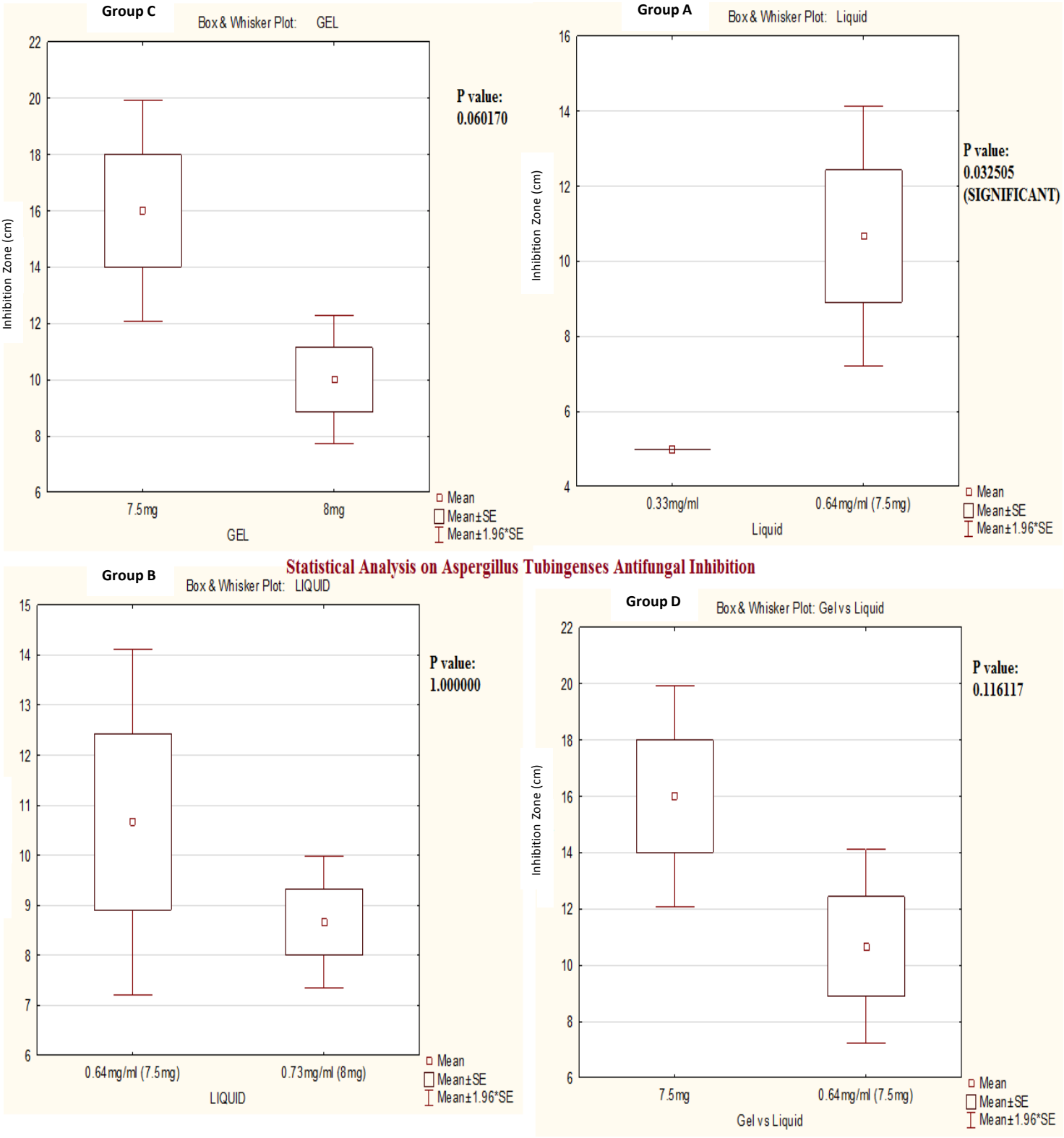


Figure 34: ANOVA test of Fungus Aspergillus Tubingensis

ANOVA tests were applied on the results obtained from antifungal testing and a box and whisker plot was made for each results.

Comparison was made between the results of:

1. Liquid formulations of Formulation A and its dilution (Group A)
2. Liquid formulations of Formulation A and Formulation B (Group B)
3. Gel formulations of Formulation A and Formulation B (Group C)
4. Liquid vs Gel formulations. (Group D)

The null hypothesis for this test claims that all populations are equal. If the P values is less than 0.005 the results are significant and we can thus reject the null hypothesis.

For the fungal strain of S27: Aspergillus Niger the P value was significant for Group A showing that the difference between the fungal zones of the dilutions was significant. For group B the P value was not significant thus, establishing the difference in fungal zones of Formulation A and Formulation B is not significant and thus it cannot be established which showed a stronger result than the other. However, P value for Group C was significant thus proving gel of Formulation A showed better antifungal properties than Formulation B. Lastly, the P value for Group D was also found to be to be significant proving that the gels showed better antifungal activity as compared to the liquid formulation.

In contrast the fungal strains of S1: Aspergillus Tubingensis showed less significant results for all the groups. The only significant results were observed in Group A within liquid dilutions of Formulation A proving that the larger dosage showed a stronger antifungal activity as compared to the less dosage. It can also be concluded that the non-significant difference in results of Group B, C and D shows that the fungal strain S1 showed more resistance to the formulation as compared to S27 and thus, the formulation showed better results against Fungal strain S27 as compared to S1.

Lastly, all the tests antifungal tests for gels as well as liquids were made one more time but this time using the drug Terbinafine Hydrochloride as the positive fungal drug to make a general comparison of the drugs antifungal capability on its own and what improvements the formation of nanoparticles brought on the antifungal activity by observing the size of the inhibition zones. Following are pictures of the results:

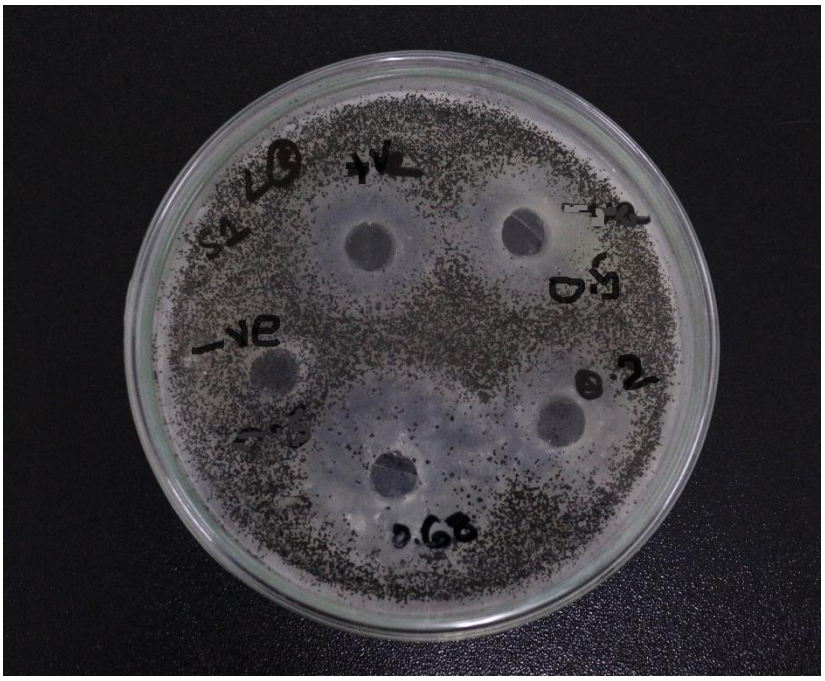


Figure 35: Image of antifungal zones against fungal strain S1 of Formulation A and its dilutions with Terbinafine Hydrochloride as the positive

In the image above plates were made of wells of 2 dilutions (0.5mg/ml, 0.2mg/ml) of liquid Formulation Aand the original formulation (0.68mg/ml) to compare activity of terbinafine hydrochlorides activity as a positive and make its comparison with Formulation A. It can be observed that on its own terbinafine hydrochloride dissolved in methanol and added to the wells shows considerable antifungal activity when compared to the nano particle formulation but the formulation shows a larger inhibition zone. Confirming that nanoparticles increase the permeation ability of the drug and help it to give a stronger antifungal response.

CHAPTER 5

CONCLUSION AND RECCOMENDATIONS

5. CONCLUSION AND RECCOMENDATIONS

The study focused on the formation of a formulation to fight against fungal infection. The drug Terbinafine Hydrochloride was chosen as the drug to undergo the process of Nanoprecipitation to form Nanocapsules of the drug. The nano particles comprised of an inner core of the drug and an outer core of the polymer HPMC-P suspended in an aqueous suspension of water and surfactant Tween 80. The formulations with the least to no precipitations and homogeneity were chosen for further characterization through SEM, FTIR, DLS and Zeta Potential studies and UV Spectrophotometry. Further in-vitro testing was conducted on the formulations in the form of In-vitro drug release studies and Encapsulation efficiency. An in-vitro antifungal assay was also conducted by making further gels of the formulations for comparison between the formulations efficiency as a liquid and formulations efficiency as a gel. The best liquid formulation was milky white in colour with no precipitation formation for the period of three months that it was observed for and the drug concentration in the formulation was 7.5mg. Through in-vitro antifungal assay it was determined that the formulation showed a stronger result in gel form as the gels are more viscous as compared to liquids.

For further recommendations for this study it is suggested to work along the established protocols and work on parameters like effect of pH on the stability of the nanoparticles as well as the antifungal properties. The study could be replicated by using another polymer and see the effect of the new polymer on the drug Terbinafine Hydrochloride. Effect of the polymer on size, zeta potential and fungal activity could be assessed. The study could also be taken further and tested on rat models or formulation of applications of the nanoparticles as water cleaning agents or antifungal nanoparticle patches for skin.

CHAPTER 6

BIBLIOGRPAHY

6. BIBLIOGRAPHY

1. A. Budhian, S.J. Siegel, K.I. Winey. (2007). Haloperidol-loaded PLGA nanoparticles: systematic study of particle size and drug content *International Journal of Pharmaceutics, 336*, 367-373.
2. AD, Russell. (2003). Similarities and differences in the responses of microorganisms to biocides. *The journal of antimicrobial chemotherapy, 52*(5), 750-763.
3. Agarwal AK, Xu T, Jacob MR, Feng Q, Li XC, Walker LA, Clark AM. (2008). Genomic and genetic approaches for the identification of antifungal drug targets. *Infectious Disorders - Drug Targets, 17*(3), 2-15.
4. Alberti I, Kalia YN, Naik A, Bonny JD, Guy RH. (2001). In vivo assessment
5. of enhanced topical delivery of terbinafne to human stratum corneum. *Journal of Controlled Release, 71*(3), 319-327.
6. Alberti, Ingo, Kalia, Yogeshvar N., Naik, Aarti, Bonny, Jean-Daniel, & Guy, Richard H. (2001). Effect of ethanol and isopropyl myristate on the availability of topical terbinafine in human stratum corneum, in vivo. *International Journal of Pharmaceutics, 219*(1–2), 11-19. doi: http://dx.doi.org/10.1016/S0378-5173(01)00616-0
7. Anbhule, S.Z. Chemate and Rahul M. (2016). Formulation and evaluation of terbinafine hydrochloride film forming emulgel. *International Journal of Drug Research and Technology, 6*(3), 164-174.
8. Balunas MJ, Kinghorn AD. (2005). Drug discovery from medicinal plants. *Life Sciences 78*(5), 431-441.
9. Beck-Sague CM, Jarvis WR (1993). Secular trends in the epidemiology of nosocomial fungal infections in the United States, 1980-1990. National Nosocomial Infections Surveillance System. *The Journal of Infectious Diseases 167*, 1247- 1251.
10. Blanco MD, Bernardo MV, Teijón C, Sastre RL, Teijón JM. (2003). Transdermal application of bupivacaine-loaded poly(acrylamide(A)-co-monomethyl itaconate) hydrogels. *International Journal of Pharmaceutics, 255*, 99-107.
11. Canilha L, de Almeida e Silva, Batista J, Solenzal AIN. (2004). Eucalyptus hydrolysate detoxification with activated charcoal adsorption or ion-exchange resins for xylitol production. *Process Biochemistry, 39*, 1909-1912.
12. Carrillo-Muñoz A.J, Giusiano G, Ezkurra P.A , Quindós G. (2006). Antifungal agents: mode of action in yeast cells. *Spanish Journal of Chemotherapy, 19*, 130-139.
13. Cevc G, Richardsen H. (1999). Lipid vesicles and membrane fusion. *Advanced Drug Delivery Reviews 38*(3), 207-232.
14. Chang, E. H., Harford, J. B., Eaton, M. A., Boisseau, P. M., Dube, A., Hayeshi, R., . . . Lee, D. S. (2015). Nanomedicine: Past, present and future - A global perspective. *Biochem Biophys Res Commun, 468*(3), 511-517. doi: 10.1016/j.bbrc.2015.10.136
15. Chen, Y. C., Liu, D. Z., Liu, J. J., Chang, T. W., Ho, H. O., & Sheu, M. T. (2012). Development of terbinafine solid lipid nanoparticles as a topical delivery system. *Int J Nanomedicine, 7*, 4409-4418. doi: 10.2147/IJN.S33682
16. Cruz M.C., Santos P.O., Barbosa A.M., de Mélo D.L., Alviano C.S., Antoniolli A.R., Alviano, & D.S., Trindade R.C. (2007). Antifungal activity of Brazilian medicinal plants involved in popular treatment of mycoses. *Journal of ethnopharmacology, 111*(2), 409-412.
17. Essa EA, Bonner MC, Barry BW. (2002). Human skin sandwich for assessing shunt role penetration during passive and iontophoretic drug and liposome delivery. *Journal of Pharmacy and Pharmacology, 54*(11), 1481- 1490.

18. F, Granier. (2000). Invasive fungal infections. Epidemiology and new therapies. *Presse Medical, 29*, 2051-2056.
19. Frankenburg, S., Glick, D., Klaus, S., & Barenholz, Y. (1998). Efficacious topical treatment for murine cutaneous leishmaniasis with ethanolic formulations of amphotericin B. *Antimicrob Agents Chemother, 42*(12), 3092-3096.
20. Gaba, Bharti, Fazil, Mohammad, Khan, Saba, Ali, Asgar, Baboota, Sanjula, & Ali, Javed. (2015). Nanostructured lipid carrier system for topical delivery of terbinafine hydrochloride. *Bulletin of Faculty of Pharmacy, Cairo University, 53*(2), 147-159. doi: 10.1016/j.bfopcu.2015.10.001
21. Gené J, Azón-Masoliver A, Guarro J, De Febrer G, Martínez A,, & C, Grau. (2001). Cutaneous infection caused by Aspergillus ustus, an emerging opportunistic fungus in immunosuppressed patients. *Journal of Clinical Microbiology*, 1134- 1136.
22. Georgopapadakou NH, Walsh TJ. (1994). Human mycoses: drugs and targets for emerging pathogens. *Science, 264*(5157), 371-373.
23. He L, Liu Y, Mustapha A, Lin M (2011). Antifungal activity of zinc oxide nanoparticles against Botrytis cinerea and Penicillium expansum. *Microbiological Research, 166*(3), 207-215.
24. Ida Idayu Muhamad, Suguna Selvakumaran, Nurul Asmak Md Lazim. (2014). Designing Polymeric Nanoparticles for Targeted Drug Delivery System (Book Chapter). Retrieved 11 December 2016, from One Central Press (OCP) http://www.onecentralpress.com/designing-polymeric-nanoparticles-for-targeted-drug-delivery-system/
25. Ishimaru K, Vystavel T, Bronsveld P, Hata T, Imamura Y, De Hosson J. (2001). Diamond and pore structure observed in wood charcoal. *Journal of Wood Science, 47*, 414-416.
26. J, Hadgraft. (2001). Skin, the final frontier. *International Journal of Pharmaceutics, 224*, 1-18.
27. Jiménez M.M., Pelletier J., Bobin M.F. and Martini M.C. (2004). Influence of encapsulation on the in vitro percutaneous absorption of octyl methoxycinnamate. *International Journal of Pharmaceutics, 272*, 45-55.
28. K.E. Geckeler, J. Stirn. (1993). Polyreaktionen – Mechanismen, Systematik, Relevanz. *Naturwissenschaften, 80*, 487-500.
29. Kalia, Yogeshvar N., Guy RH. (2001). Modeling transdermal drug release. *Advanced Drug Delivery Reviews, 48*, 159-172.
30. Karim Miladi, Sana Sfar, Hatem Fessi, Abdelhamid Elaissari. (2016). Nanoprecipitation Process: From Particle Preparation to In Vivo Applications. In G. P. Christine Vauthier (Ed.), Polymer Nanoparticles for Nanomedicines (pp. 17-53): Springer International Publishing Switzerland.
31. Kim D.G., Jeong Y.I., Choi C., Roh S.H., Kang S.K., Jang M.K. and, & J.W, Nah. (2006). Retinol-encapsulated low molecular water-soluble chitosan nanoparticles. *International Journal of Pharmaceutics, 319*, 330-338.
32. Kim, Il Hyuk, Park, Jung Hwan, Cheong, In Woo, & Kim, Jung Hyun. (2003). Swelling and drug release behavior of tablets coated with aqueous hydroxypropyl methylcellulose phthalate (HPMCP) nanoparticles. *Journal of Controlled Release, 89*(2), 225-233. doi: 10.1016/s0168-3659(03)00089-0
33. Lboutounne H, Faivre V, Falson F, Pirot F. (2004). Characterization of transport of chlorhexidine-loaded nanocapsules through hairless and wistar rat skin. *Skin Pharmacology and Physiology, 17*, 176-182.

34. Lince F, Marchisio DL, Barresi AA. (2008). Strategies to control the particle size distribution of poly-e-caprolactone nanoparticles for pharmaceutical applications. *Journal of Colloid and Interface Science 322*(2), 505-515.
35. M., Foldvari. (2000). Non-invasive administration of drugs thourough the skin: Challenges in delivery system design. *Pharmaeutical Science and Technology Today, 3*, 417-425.
36. M. Zohri, T. Gazori, S. Mirdamadi, A. Asadi & I. Haririan. (2009). Polymeric NanoParticles: Production, Applications and Advantage. *The Internet Journal of Nanotechnology, 3*(1).
37. Manoj Singh, Manish Kumar, R. Kalaivani, S. Manikandan, A. K. Kumaraguru. (2013). Metallic silver nanoparticle: a therapeutic agent in combination with antifungal drug against human fungal pathogen. *Bioprocess and Biosystems Engineering, 36*(4), 407-415.
38. Martinez-Rossi, N.M., Peres, N.T., Rossi, A Mycopathologia (2008). Antifungal resistance mechanisms in dermatophytes. *166*, 369–383.
39. Morganti P, Ruocco E, Wolf R, Ruocco V. (2001). Percutaneous absorption and delivery systems. *Clinics in Dermatology, 19*(4), 489-501.
40. Moser K, Kriwet K, Naik A, Kalia YN, Guy RH. (2001). Passive skin penetration enhancement and its quantification in vitro. *European Journal of Pharmaceutics and biopharmaceutics, 52*(2), 103-112.
41. Murat S, Cengiz Uzun, Olgun Gu¨ven. (2000). Controlled release of antifungal drug terbinafine. *International Journal of Pharmaceutics*, 149-157.
42. Nicola Cioffi, Luisa Torsi, Nicoletta Ditaranto, Giuseppina Tantillo, Lina Ghibelli, Luigia Sabbatini, Teresa Bleve Zacheo, Maria D'Alessio, P. Giorgio Zambonin, and Enrico Traversa. (2005). Copper Nanoparticle/Polymer Composites with Antifungal and Bacteriostatic Properties. *Chemistry of Materials, 17*(21), 5255-5262.
43. Ozcan, I., Abaci, O., Uztan, A. H., Aksu, B., Boyacioglu, H., Guneri, T., & Ozer, O. (2009). Enhanced topical delivery of terbinafine hydrochloride with chitosan hydrogels. *AAPS PharmSciTech, 10*(3), 1024-1031. doi: 10.1208/s12249-009-9299-x
44. Pathan IB, Setty CM. (2009). Chemical penetration enhancers for transdermal drug delivery systems. *Tropical Journal of Pharmaceutical Research, 8*(2), 173-179.
45. Paulo CS, Vidal M, Ferreira LS. (2010). Antifungal nanoparticles and surfaces. *Biomacromolecules, 11*(10), 2810-2817.
46. Pison, U., Welte, T., Giersig, M., & Groneberg, D. A. (2006). Nanomedicine for respiratory diseases. *Eur J Pharmacol, 533*(1-3), 341-350. doi: 10.1016/j.ejphar.2005.12.068
47. Rajeshkumar R, Sundararaman M. (2012). Emergence of Candida spp. and exploration of natural bioactive molecules for anticandidal therapy--status quo. *Mycoses, 55*(3), 60-73.
48. Ravi, S., Peh, K. K., Darwis, Y., Murthy, B. K., Singh, T. R., & Mallikarjun, C. (2008). Development and characterization of polymeric microspheres for controlled release protein loaded drug delivery system. *Indian J Pharm Sci, 70*(3), 303-309. doi: 10.4103/0250-474X.42978
49. Rojas JJ, Ochoa VJ, Ocampo SA, Muñoz JF. (2006). Screening for antimicrobial activity of ten medicinal plants used in Colombian folkloric medicine: a possible alternative in the treatment of non-nosocomial infections. *BMC Complementary and Alternative Medicine, 6*(2).
50. Ryder, N. S. (1992). Terbinafine: Mode of action and properties of the squalene epoxidase inhibition. *British Journal of Dermatology, 126*(s39), 2-7. doi: 10.1111/j.1365-2133.1992.tb00001.x

51. Saadia Ahmed Tayel, Mohamed Ahmed El-Nabarawi, Mina Ibrahim Tadros and Wessam Hamdy Abd-Elsalam. (2013). Positively Charged Polymeric Nanoparticle Reservoirs of Terbinafine Hydrochloride: Preclinical Implications for Controlled Drug Delivery in the Aqueous Humor of Rabbits. *AAPS PharmSciTech, 14*(2), 782-793. doi: 10.1208/s12249-013-9964-y
52. Samrat, K., Nikhil, N. S., Karthick Raja Namasivamyam, S., Sharath, R., Chandraprabha, M. N., Harish, B. G., . . . Kashyap, Rakesh G. (2016). Evaluation of improved antifungal activity of fluconazole – silver nanoconjugate against pathogenic fungi. *Materials Today: Proceedings, 3*(6), 1958-1967. doi: 10.1016/j.matpr.2016.04.097
53. Sawangphruk M, Srimuk P, Chiochan P, Sangsri T, Siwayaprahm P. (2012). Synthesis and antifungal activity of reduced graphene oxide nanosheets. *Carbon, 50*, 5156- 5161.
54. Schmidt B, Ribnicky DM, Poulev A, Logendra S, Cefalu WT, Raskin I. (2008). A natural history of botanical therapeutics. *Metabolism: clinical and experimental, 57*, S3-9.
55. Scorzoni, L., Sangalli-Leite, F., de Lacorte Singulani, J., de Paula, E. Silva A. C., Costa-Orlandi, C. B., Fusco-Almeida, A. M., & Mendes-Giannini, M. J. (2016). Searching new antifungals: The use of in vitro and in vivo methods for evaluation of natural compounds. *J Microbiol Methods, 123*, 68-78. doi: 10.1016/j.mimet.2016.02.005
56. Shim J., Kang H.S., Park W., Han S., Kim J. and Chang I. (2004). Transdermal delivery of mixnoxidil with block copolymer nanoparticles. *Journal of Controlled Release, 97*, 477-484.
57. Stephanie Hornig, Thomas Heinze, C. Remzi Becer and Ulrich S. Schubert. (2009). Synthetic polymeric nanoparticles by nanoprecipitation. *Journal of Materials Chemistry, 19*, 3838–3840.
58. Terbinafine Hydrochloride. (2017). 2017, from https://pubchem.ncbi.nlm.nih.gov/compound/5282481
59. Thiruganesh R, Uma Devi SK. (2010). olid lipid nanoparticle and nanoparticle lipid carrier for controlled drug delivery – a review of state of art and recent advances. *International Journal of Nanoparticles, 3*(1), 32-52.
60. Ting W.W., Vest C.D. and Sontheimer R.D. (2004). Review of traditional and novel modalities that enhance the permeability of local therapeutics across the stratum corneum. *International Journal of Dermatology, 43*, 538-547.
61. Trejo WH, Bennet RE. (1963). Streptomyces nodosus sp. n., the amphotericin-producing organism. *Journal of Bacteriology, 85*, 436-439.
62. Ulrich P., Tobias W., Michael G., David A G. (2006). Nanomedicine for respiratory diseases. *European Journal of Pharmacology, 533*, 341 - 350.
63. Vidyavathi Maravajhala, Sandya Papishetty and Sarika Bandlapalli. (2012). Nanotechnology in Development of drug delivery system. *International Journal of Pharmaceutical Sciences and Research, 3*(1), 84-96.
64. Wavikar, P., & Vavia, P. (2013). Nanolipidgel for enhanced skin deposition and improved antifungal activity. *AAPS PharmSciTech, 14*(1), 222-233. doi: 10.1208/s12249-012-9908-y
65. Wiederhold N.P., Patterson T.F . (2015). What's new in antifungals: an update on the in-vitro activity and in-vivo efficacy of new and investigational antifungal agents. *Current opinion in infectious diseases, 28*(6), 539-545.
66. Wim H De Jong, Paul JA Borm. (2008). Drug Delivery and nanoparticles: Application and hazards. *International Journal of Nanomedicine, 3*(2), 133-149.
67. Y, Masayuki. (2005). Drug targeting with nano-sized carrier systems. *Journal of Artificial Organs, 8*, 77-84.

68. Yadav H.K.S, Nagavarma B.V. N, Ayaz A, Vasudha L.S, Shivakumar H.G. (2012). Different Techniques for Preparation Of Polymeric Nanoparticles -A Review. *Asian Journal of Pharmaceutical and Clinical Research, 5*, 16-23.
69. Yokoyama, Masayuki. (2005). Drug targeting with nano-sized carrier systems. *Journal of Artificial Organs, 8*(2), 77-84. doi: 10.1007/s10047-005-0285-0
70. Zain, Mohamed E. (2011). Impact of mycotoxins on humans and animals. *Journal of Saudi Chemical Society, 15*(2), 129-144.