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Topical treatment of human Dermatophytoses by new formula of Amphotericin B using of animal model

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By

Falah Hasan Obayes AL-Khikani

B.Sc. College of medical and health technology Baghdad (2013)

Supervised By

Prof. Ali Abdul Hussein S. AL-Janabi

Dr. Luay Mohamed D. Mahde AL-Rubaeai

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فَوَجَدَا عَبْدًا مِّنْ عِبَادِنَا آتَيْنَاهُ رَحْمَةً مِّنْ عِندِنَا وَعَلَّمْنَاهُ مِن لَّدُنَّا عِلْمًا

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Certification

We certify that thesis was prepared under our supervision at the College of Medicine, University of Kerbala, as a partial fulfillment of the requirements for the Degree of Master of Science in Medical Microbiology.

Signature:

Ali Abdul Hussein S. AL-Janabi

Title: professor

Address:

Dept. of microbiology,

College of medicine, University

of Kerbala

Signature:

Luay Mohamed D. ALRubaeai

Title: dermatologist doctor

Address:

Al-Imam Al- Hussein Medical

city hospital

Chair of Microbiology Department

In view of the available recommendation, I forward this thesis for debate by the examining committee

Head of department

Signature:

Name: Asst. Prof. Ali Mansour Al-Ameri
College of medicine / university of kerbala

:: Committee certification ::

We, the examiner committee certify that we´ve read the M.Sc. thesis entitled:

Topical treatment of human Dermatophytoses by new formula of Amphotericin B using of animal model

We have examined the student (**Falah Hasan Obayes AL-Khikani),** in its contents. In our opinion it is meets the standards of thesis for the degree of Masters in Medical Microbiology and Immunology.

Prof. Majid Kadim Abood

College of Education, University of Al- Qadisiyah

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Assist Prof. Ali Tariq Abdul Hussein

College of Medicine, University of Karbala

Member

Dr. Ahmed Mohammed Al- mothfer

College of Medicine, University of Kufa

Member

Prof. Ali Abdul Hussein S. AL-Janabi

College of Medicine, University of Karbala

Member-Supervisor

Dr. Luay Mohamed D. AL-Rubaeai

Al-Imam Al-Hussein Medical city, Karbala

Member- Supervisor

Approved by the College of Medicine – Karbala University

Prof. Hassan Ali Abood

Dean

Dedication

To the most precious person in my life, who encouraged me to reach the beach of Success……...My big brother Mudher

The person who had sacrificed her health in my upbringing and education…… My true love mother

To my pride and honor of the absent … the present, my God has mercy upon his soul…..…... My father

To my dear hero brother …… …… Eng. Abed alwarith

To the kindest hearts …… My sisters

To the sweet heart ……...…... My wife

To the twilight stars ……. Precious children (Temojeen Falah and Muetaz Mudher)

The absent person who,but always present ……

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Summary

There is actually no part of the world can be cleared from infection with dermatophytosis. The skin, hair and nail of all types of mammalian, including the human are under the risk to develop dermatophytosis. The disease is mainly caused by different species of dermatophytes within the cutaneous layer of the skin. Several topical and systemic antifungal drugs are used for treatment of dermatophytosis. Amphotericin B (AmB) is widely intravenous used for treatment of systemic fungal infection. Topical formula of AmB is still under experimental level.

In this study, topical Amphotericin B cream 1% (AmB) was prepared to use as treatment for dermatophytosis in animal and human. Rabbits were chosen to be an animal model for treatment by new preparation of AmB cream. A total of 12 healthy rabbits were infected by clinical isolate of *Trichophyton mentagrophytes*. Rabbits were divided into four groups with 3 rabbits in each. First group was treated twice daily with prepared AmB cream 1%, second group with twice daily clotrimazole cream 1%; third group with cream only; and forth group was left without treatment as a control. The curative time of treated animal by AmB was found shorter (4-6 days) than by clotrimazole cream (14-28 days) with significant differences ($p< 0.05$).

Two groups of 12 patients with dermatophytosis were involved in this study. First group of six patients was treated with prepared topical AmB 1%, while other six participants were treated with clotrimazole cream 1%. As with treated animals, the curative period of AmB was shorter (10 day) compared with clotrimazole cream (14-21 days) with significant differences ($p< 0.05$).

Tinea corporis was the most prevalence type of dermatophytosis (91.66%) in involved patients, followed by tinea barbae (8.33%). *Microsporum canis* was more frequently isolated (83.32%) from patients with tinea corporis, while *T. mentagrophytes* was isolated from one patient (16.66%). Males at age range 20-50 years were more infected by dermatophytosis than females (18-52 years). Clinical features of dermatophytosis ranged from mild to severe for most involved patients in this study. Occupation type of males and females was variable between students and other type of jobs. Some of involved patients were in contact with animals and birds.

List of contents

List of Figures

List of Tables

List of Abbreviations

ABCD	Amphotericin B colloidal dispersion
ABLC	Amphotericin B lipid complex
AIDS	Acquired immunodeficiency syndrome
AmB	Amphotericin B
AmB-np	Amphotericin B nanoparticles
AmBUDL	Amphotericin B ultradeformable liposomes
D-AmB	Deoxycholate Amphotericin B
FDA	Food and drug administration
FPA	Free polyaggregated Amphotericin B
HIV	Human immunodeficiency virus
K^+	Potassium ion
L-AmB	Liposomal amphotericin B
MFC	Minimum fungicidal concentration
Mg^+	Magnesium ion

MIC	Minimum inhibitory concentration
rRNA	Ribosomal ribonucleic acid
SLNs	Solid lipid nanoparticles
SGA	Sabouraud’s glucose agar
SGB	Sabouraud’s glucose broth

Chapter One

Introduction

1. Introduction:

Dermatophytes are filamentous fungi naturally living on keratinous materials found in soil (Zhan and Liu**,** 2017). They include about 40 different species which can be primary diagnosed by their morphological characters. All of these species are involved in three genera; Trichophyton, Microsporum and Epidermophyton (Havlickova *et al*., 2008; Metin and Heitman, 2017).

Dermatophytosis or tinea is mainly caused by dermatophytes (Tampieri, 2004). It is considered a prevalent skin diseases worldwide (Bouchara *et al*., 2017). Tinea is predominant in about 20-25% over worlds' populations (Havlickova *et al*., 2008; Lopes *et al*., 2017). Moisture and warm conditions are the most suitable factors to wide distribution of dermatophytosis in tropical countries (Havlickova *et al*., 2008). This epidemiological distribution may change with migration, lifestyle, immunosuppressive state, drug therapy and socioeconomic conditions (Havlickova *et al*., 2008; Ameen, 2010). Dermatophytosis can occur in either of the human and animals (Seker and Dogan, 2011; Moretti *et al*., 2013; Pin, 2017). Thus, usage of animal model will consider a primary step for *in vivo* evaluation of any new drug for treatment of dermatophytosis (Cambier *et al*., 2017).

Amphotericin B (AmB) which is belonged to the polyene group has a wide spectrum *in vitro* and *in vivo* antifungal activity (Diekema *et al*., 2003; Stone *et al*., 2016). Pore formation in the fungal plasma membrane is the most acceptable mechanism of antifungal action of AmB (Hartsel *et al*., 1993; Shimizu *et al*., 2010; Mesa-Arango *et al*., 2012; Gray *et al*., 2012; Stone *et al*., 2016). Deoxycholate AmB (D-AmB) is an old form of AmB used for the treatment of various systemic fungal infections (Stone *et al*., 2016). It has a diverse side effects represented by nepherotoxicity (Laniado-Laborín and

Cabrales-Vargas, 2009) and influence on the liver metabolic capacity (Inselmann *et al*., 2002). Thus, new lipid formulas were developed in 1990 (Torrado *et al*., 2008). Recently, the main available lipid formulas of AmB, including liposomal amphotericin B (L-AmB), Amphotericin B lipid complex (ABLC) and Amphotericin B colloidal dispersion (ABCD) (Stone *et al*., 2016; Steimbach *et al*., 2017; Serrano and Lalatsa, 2017).

The recent studies have been tried to prepare a topical formula of AmB in order to limit its side effects. Most of these studies focused on topical preparation of AmB as eye drop (Morand *et al*., 2007) or gel (Ruiz *et al*., 2014) or solution (Trasmonte *et al*., 2012) or as nanoparticles drug (Sanchez *et al*., 2014). However, treatment with topical AmB may not always give satisfied results as with ordinary forms of this drug in treatment of fungal infection.

Aims of the study

1- To detect the ability of rabbits to be animal model for infected with dermatophytes.
2- To Prepare of amphotericin B cream 1% as topical treatment of dermatophytosis.
3- To evaluate therapeutic ability of prepared AmB cream for treatment of animals and humans with dermatophytosis.

Chapter Two

Review of literatures

2. Review of literatures:

2. 1. Dermatophytes:

Dermatophytes are a special group of keratinous fungi which have the ability to live on keratin-rich materials that found in soil or in the human or animals tissues such as skin, hair, and nail (Zhan and Liu**,** 2017). They involve about 40 different species included within three most important genera of Trichophyton, Microsporum and Epidermophyton (Tampieri, 2004; Havlickova *et al*., 2008; Metin and Heitman, 2017). Based on morphological characters, all of these genera consider anamorphic form of the class Hyphomycetes of the phylum Deutromycota (imperfect fungi) (Weitzman and Summerbell, 1995). Sexual stage (teleomorphe) for some of Trichophyton and Microsporum genera is also discovered to make them included within Arthrodermataceae of ascomycetes (Aneja *et al*., 2013). However, molecular assays which are depending on analysis of rRNA sequences confirmed that all dermatophytes are a cohesive group, with no clear distinction between the three genera (White *et al*., 2014).

The difference in macroconidia characters is the old morphological feature used to differentiate between three genera of dermatophytes. The species of Trichophyton genus produce smooth, thin wall, and 1-12 septa macroconidia which are borne singly or in cluster with clavate, fusiform or cylindrical shape. Whereas, the macroconidia of Microsporum genus have a thick rough wall with asperulate, echinulate or verrucose surface and spindle, fusiform, or egg shape with 1-15 septa. Fungal species of Epidermophyton genus can produce a broad clavate macroconidia with moderately thick wall and 1-9 septa and usually borne as a single or cluster conidia (Weitzman and Summerbell, 1995).

Dermatophytes can classified according to the location in the environment or route of transmission into three groups, anthropophilic (transmitted from human to human), zoophilic (transmitted from animals to human), and geophilic (transmitted from soil to human) (Andrews and Burns, 2008; Panthagani and Tidman, 2015). The phylogeny of dermatophytes is more influence by the environment location of these fungi. Sexual reproduction is very clear observed among geophilic group and some of zoophilic, while it very rare observed among anthropophilic group (Metin and Heitman, 2017).

2. 2. Dermatophytosis:

There are approximately 100,000 species of fungi from a millions species of fungi on the earth have the ability to cause diseases for human and animals, especially in the temperate and tropical countries (Havlickova *et al*., 2008). Dermatophytes are an important group of pathogenic fungi causing skin diseases worldwide (Bouchara *et al*., 2017). They attend to infect the keratinized tissues such as cutaneous skin layer, hair, and nail (Havlickova *et al*., 2008; Moriarty *et al*., 2012; Panthagani and Tidman , 2015; Bouchara *et al*., 2017). Dermatophytoses or tinea is the name of the disease caused by dermatophytes (Tampieri, 2004). This disease causes chronic morbidity with a high prevalence distribution in the entire world (Moriarty *et al*., 2012; Santosh *et al*., 2015). The predominance of dermatophytosis is about 20-25% from all total worlds' population (Havlickova *et al*., 2008; Lopes *et al*., 2017). It takes a different pattern of infection in all of the world which reflect a variable geographic distribution of this disease (Ameen, 2010). Moisture and warm conditions are the most encouraged factors for development of dermatophytoses in tropical countries (Havlickova *et al*., 2008). Other factors,

including increasing sweating result from outdoor physical human activities in hot weather and low degree of hygiene are also associated with the prevalence of dermatophytosis (Jena *et al*., 2018). However, epidemiology of such disease has changed due to lifestyle, migration, socioeconomic conditions, drug therapy and immunosuppressive state (Havlickova *et al*., 2008; Ameen, 2010).

Dermatophytosis or tinea can be found on the skin of different parts of the human body which make it takes various names based on the infected area such as tinea pedis on the feet, tinea unguium on the nails, tinea capitis on the scalp, tinea cruris on the groin, and tinea corporis on the body (Andrews and Burns, 2008). A lesion of tinea can be caused by a single species of dermatophyte or by many species in some cases (Singla *et al*., 2019). Additionally, a single species of dermatophytes can caused different types of tinea (Jena *et al*., 2018). Tinea corporis is the more common type of tinea which is mostly caused by *Trichophyton* species (Havlickova *et al*., 2008; Andrews and Burns, 2008), while tinea capitis most frequent caused by *T. violaceum, T. tonsurans* and *M. canis* (Zhan and Liu, 2017). From all species of dermatophytes, *Trichophyton rubrum, M. canis, T. interdigitale* (*mentagrophytes var. interdigitale), T. tonsurans, T. verrucosum* and *M. audouinii* are the most account for dermatophytosis worldwide (Havlickova *et al*., 2008). The investigation for these fungi is very important in diagnosis, treatment and differentiation from other clinical skin diseases (Kaur *et al*., 2019). *Trichophyton rubrum* is the predominant isolates from human followed by *Trichophyton mentagrophytes* (Surendran *et al*., 2014; Bhagra *et al*., 2014; Jena *et al*., 2018; Kadhim, 2018; Adesiji *et al*., 2019). This is clear in Europe when a high incidence of *T. rubrum* infection was recorded, while *T. mentagrophytes* was higher incidence in Asia (Havlickova *et al*., 2008).

The general clinical features of tinea on the human body are represented by gradually appearance of annular erythematous lesion with central healing tendency (Libon *et al*., 2017). Scaling, pustules, itching, inflammation and hair and nail loss are also characters of most dermatophytosis infection (Bouchara *et al*., 2017).

The ability of dermatophytes to produce various proteins or enzymes plays an important role to invade keratineous skin layers (Martinez-Rossi *et al*., 2017). Keratinases, adhesins, lipases, phosphatases, DNAses and non-specific proteases are important enzymes give the fungi the ability to attach and penetrate the stratum corneum of the skin, overcome host immune system and scavenge nutrients (Martinez-Rossi *et al*., 2012). Keratinase and phospholipase found to be produced by 96% of 234 clinical dermatophytes isolates, while gelatinase and elastase produced from 14% and 23% of isolates, respectively (Gnat *et al*., 2018). The acidic nature of the skin stimulate dermatophyte to produce sensing transcription factors such as PacC and Hfs1 to raise fungi adapting to this acidic pH and give the time to increase pH value after keratin degradation for elevate protease enzymes activity (Martinez-Rossi *et al*., 2017).

2. 3. Dermatophytosis in animal:

Dermatophytes have the ability to cause dermatophytoses in different types of animals (domestic and wild) as well as in the human (Radentz, 1991; Seker and Dogan, 2011; Moretti *et al*., 2013; Pin, 2017). The zoophilic group of dermatophytes that mainly infected animals can easily cause infection in the human with a progressive lesion than that caused by anthropophilic members of dermatophytes (Radentz, 1991). Otherwise, the human can

sometime become a source for infecting other farm or wild animals as noted in the laboratory or other work places (Moretti *et al*., 2013). Dermatophytoses in animals may have a significant correlation with the age of the animal, but not with the gender or with the season even though that dogs is highly infected in winter and spring compared with cats that mostly infected in autumn, summer and spring (Seker and Dogan, 2011). A single or multiple follicular lesions is the most clinical features of dermatophytoses in animals that may associate with the hair loss, crusting, scaling and erythrema (Pin, 2017).

Trichophyton mentagrophytes and *Microsporum canis* are the common causative zoophilic agents of dermatophytoses in animals (Radentz, 1991). Rabbits and Guinea pigs are mostly infected by *T. mentagrophytes* as observation of a positive culture in 72.3% and 91.6% of them, respectively (Kraemer *et al*., 2012). Therefore, the great number of infected rabbits (15 from 19 rabbits) with *T. mentagrophytes* can consider a risk factor to their owners, particularly children (Krämer *et al*., 2012). Otherwise, adult rabbits can become a carrier to dermatophytes (Moretti *et al*., 2013). However, the lesion of dermatophytoses in rabbit revealed alopecia with crusts or yellowish-white dry scales chiefly on the head and can spread to other parts of the rabbit body (Krämer *et al*., 2012; Moretti *et al*., 2013). *M. canis* is the most causative agent of dermatophytoses in cats and dogs compared with *T. mentagrophytes* (Seker and Dogan, 2011; Proverbio *et al*., 2014). From 15 cats with dermatophytoses, 13 revealed positive results for *M. canis*, while only 2 with *T. mentagrophytes* (Proverbio *et al*., 2014). Meanwhile, five-fold infected dogs than cats are frequently caused by *T. mentagrophytes* (Seker and Dogan, 2011).

2. 4. Amphotericin B (AmB):

Amphotericin B (AmB) is one of polyene group that have a wide antifungal activity against most types of yeasts, molds and a protozoan *Leishmania* spp. (Moen *et al*., 2009; Stone *et al*., 2016). It's naturally produced by soil actinomyces, *Streptomyces nodosus* (Hamill, 2013). The main characters of AmB are its yellowish color and aggregation nature with a low solubility in water and most organic solvents, but can increase solubility at pH under 2 or more than 11 (Torrado *et al*., 2008). Over more than 50 years, AmB still prefer to use with a high efficiency in clinical medicine to treatment various fungal infection in the human body (Baginski and Czub, 2009; Volmer *et al*., 2010). Deoxycholate AmB (D-AmB) is the first form of AmB developed in 1950 to use against systemic fungal infections (Stone *et al*., 2016). It quickly approved to use clinically by FDA in 1958 in spite of unknown its structure due to its broad spectrum antifungal activity (Volmer *et al*., 2010). In 1958, an intravenous formula of sodium D-AmB solution was presented in the markets at the name Fungizone-Squibb (Mesa-Arango *et al*., 2012). Low fungal resistance and broad spectrum antifungal activities are the most valuable pharmaceutical characters encourage continuous usage of AmB (Lanternier and Lortholary, 2008). Although wide clinical use of AmB for more than five decades, fungal resistance is rarely recorded until now compared with other antifungal agents (Ghannoum and Rice, 1999; Cannon *et al*., 2007; Mesa-Arango *et al*., 2012; Gray *et al*., 2012). As with any drug, AmB has adverse effects that may prevent it used even in the presence of serious systemic fungal infection. Nepherotoxicity is the major side effects yield from chronic used of more than 35 mg/day of AmB (Laniado-Laborín and Cabrales-Vargas, 2009). It also influence on the liver metabolic capacity

through interaction with hepatic cytochrome P450 (Inselmann *et al*., 2002). However, the old formula of AmB that contain deoxycholate have more nephrotoxicity effects than new lipid formula developed in 1990 which release low free AmB concentration in serum (Torrado *et al*., 2008).

2. 5. Mechanism of action of AmB:

There is no clear mechanism of action is confirmed to explain the antifungal effect of AmB although it used for more than 50 decades. The more acceptable one is the activity of AmB to bind with the ergosterol of the fungal plasma membrane causing dysfunction of it through forming ion pore channel (Hartsel *et al*., 1993; Shimizu *et al*., 2010; Mesa-Arango *et al*., 2012; Gray *et al*., 2012; Stone *et al*., 2016). Pore formation will lead to inhibition of fungal glycolysis and rapid efflux of K^+, and Mg^+ ions inside fungal cells which increase the acidity of these cells causing cell death (Hamill, 2013). The higher affinity of AmB to bind with ergosterol than with mammalian cholesterol and its bigger molecule size to make more membrane damage and conduct high amount of ion can support this mechanism (Baginski *et al*., 2002). Two main domains in the chemical structure of AmB molecule play a role in pore forming in fungal plasma membrane, including hydrophobic (hydrocarbon chain) that forming a pore and hydrophilic (polyhydroxyl chain) that facing the interior of the pore (Hamill, 2013).

Oxidative stress is another mechanism of AmB action against fungi through production of free radicals inside the fungal cells (Sangalli-Leite *et al*., 2011; Mesa-Arango *et al*., 2012). The oxidation effect of AmB will lead also to form superoxide anion and oxygen depletion in which effect on the organism cell pathways (Haido and Barreto-Bergter, 1989). Moreover, AmB

has the ability to induce proinflammatory immune response due to its immunomodulatory properties and this will give the infected individual, especially those with immunocompromised state, another protective process against fungal infection (Mesa-Arango *et al*., 2012).

2. 6. Pharmaceutical formulas of AmB:

The adverse effects of D-AmB, especially nephrotoxicity, infusion reaction and dose limitation, try to limit by development new formulas with remain the same antifungal activity (Hamill, 2013). Three lipid formulas, including liposomal amphotericin B (L-AmB), amphotericin B lipid complex (ABLC) and amphotericin B colloidal dispersion (ABCD) are became more reliable to use with less side effect (Dupont, 2002; Herbrecht *et al*., 2003; Hamill, 2013; Stone *et al*., 2016; Steimbach *et al*., 2017; Serrano and Lalatsa, 2017). Although all of these formulas contain AmB, they have different properties such as reticuloendothelial clearance, size, visceral diffusion and shape (Dupont, 2002). This various properties will also associated with other characters to determine the antifungal activity of each formula such as type of infection, time of therapy starting, required dose, toxicity level, tissue location and retention, and pharmacokinetic properties (Adler-Moore and Proffitt, 2008). Thus, usages of new AmB formula will add another choice for treatment of different fungal infection even in patients with renal impairment and conventional AmB failure (Herbrecht *et al*., 2003). This choice is mainly depended on the low infusion- related toxicity, especially for L-AmB, and possibly ABLC (Hamill, 2013).

Treatment with AmB still consider the first choice against systemic fungal infection such as cryptococcosis (Cryptococcal meningitis), aspergillosis,

invasive candidiasis and other lethal opportunistic mycosis diseases as with zygomycosis and fusariosis (Dupont, 2002; Adler-Moore and Proffitt, 2008). Liposomal AmB is more prefer formula of AmB to treat brain fungal infection due to its high penetration through brain membrane and low toxicity compared with other formulas (Adler-Moore and Proffitt, 2008). However, various AmB formulas have different antifungal activity as mentioned by clinical utilization as related to the site of infection and the immune state of infected individuals (Torrado *et al*., 2008).

The usual dose of AmB should be 3-5 mg/kg/day and the effective of this dose may vary from one formula to another (Dupont, 2002). The route of AmB administration is parenterally because of its low oral bioavailability (0.2–0.9%) (Serrano and Lalatsa, 2017). However, the lipid formulas are quit more expensive than old one (Herbrecht *et al*., 2003).

2. 7. Type of AmB formulas:

2. 7. 1. Deoxycholate Amphotericin B (D-AmB)

Deoxycholate Amphotericin B (D-AmB) is the first discovered formula of AmB in 1950 results from mixing sodium deoxycholate with AmB and used to treatment of systemic fungal infections (Stone *et al*., 2016). The mixture is consisted of AmB to deoxycholate ratio of 1:2 (Torrado *et al*., 2008). The antifungal efficiency of D-AmB is always concomitant with more serious dose-related nephrotoxicity side effects (Laniado-Laborín and Cabrales-Vargas, 2009). This toxic effect limited the maximal tolerated dose of D-AmB to 0.7–1.0 mg/kg/day which is less effective to treatment systemic fungal infection, especially in immunocompromised persons (Hamill, 2013).

2. 7. 2. Liposomal amphotericin B (L-AmB)

Liposomal amphotericin B (L-AmB) is more development formula of AmB that designed to reduce the side effects of D-AmB (Stone *et al*., 2016). It presented to the European market in 1989 and approved by FDA as first drug to treat visceral leishmaniasis in August 1997 (Torrado *et al*., 2008). Its structure composed of spherical vesicles that distinguished by lipid bilayer surround aqueous core (Stone *et al*., 2016). This small unilamellar liposome structure which has 60-70 nm diameters is also regarded as particular sort of colloidal system that increase serum half-time of AmB (Torrado *et al*., 2008). The usual dose of L-AmB is 3-6 mg/kg/day and can be remained at high concentration in plasma by the effect of its negative charge, small size as well as it avoids ingestion by the mononuclear phagocytic cell (Hamill, 2013). The commercial name of L-AmB is Ambisome® (Torrado *et al*., 2008).

The L-AmB has proved to be effective against a wide range of systemic fungal infection that caused by opportunistic fungi such as candidiasis, cryptococcal meningitis in HIV and febrile neutropenia patients, disseminated histoplasmosis, life threatening mucormycosis and invasive aspergillosis (Moen *et al*., 2009; Stone *et al*., 2016). The pharmacokinetic of L-AmB started when the liposomal vesicle become in contact with fungal element in infection site, then release AmB from holding vesicle to attach with the ergosterol of fungal plasma membrane and destroy it (Stone *et al*., 2016). The liposomal structure also has an important role to reduce the nephrotoxicity effects of AmB when it used alone (Moen *et al*., 2009), but it also required monitoring after 9 days of usage from the management beginning (Kato *et al*., 2018). However, utilizing of L-AmB is also restricted by its expensive cost (Moen *et al*., 2009).

2. 7. 3. Amphotericin B lipid complex (ABLC)

The commercial name of Amphotericin B lipid complex is Abelcet® which consists of two phospholipids and AmB in 1:1 molar ratio with a diameter of 2-5 µm of ribbon-like shape (Torrado *et al*., 2008). The large size of ABLC make it easily ingested by macrophage and deposit in organs rich with this cells such as spleen and liver and also facility the clearance of ABLC concentration from plasma (Hamill, 2013). However, treatment with ABLC appeared low risks for kidney damage and more concentration in lung than other types of AmB, but showed risks of hepatic disorders (Torrado *et al*., 2008). The usual dose of ABLC is 5 mg/kg/day (Hamill, 2013).

2. 7. 4. Amphotericin B colloidal dispersion (ABCD)

Amphotericin B colloidal dispersion (ABCD) which found under a commercial name Amphotec® is characterized by its content of equal molar concentrations of cholesterol sulfate (Torrado *et al*., 2008). The diverse effects of ABCD usage are quietly similar to that of D-AmB, but it differs by quickly removing from the plasma by macrophage ingestion (Hamill, 2013).

2. 8. Amphotericin B for treatment of human dermatophytosis:

Invasive systemic fungal infections recently consider the major cause of morbidity and mortality in immunocompromised individuals who have an immunodeficiency conditions such as those with AIDS, transplant recipients or tumor patients receiving immunosuppressive chemotherapy (Torrado *et al*., 2008). The *in vitro* and *in vivo* usage of AmB reveals a broad spectrum activity against various fungi. It found to *in vitro* inhibit the growth of 89% of 448 clinical isolates molds at ≤1 µg/ml (Diekema *et al*., 2003). The minimum inhibitory concentration (MIC) of AmB is usually required less value to

inhibit the mold growth than for minimum fungicidal concentration (MFC) as noted with some mold such as *Trichoderma longibrachiatum* (MIC; 0.87, MFC; 5 µg/ml) and *Rhizopus arrhizus* (MIC; 0.36, MFC; 2.2 µg/ml) (Espinel-Ingroff, 2001). Synergism with other antifungal agents could also increase AmB activity against pathogenic fungi as the combination with flucytosine against melanized fungi of Chaetothyriales order that cause primary cerebral infections (Deng *et al*., 2016). However, inhibition of yeasts required less concentration of AmB as MIC (0.25 to 1.0 µg/ml) compared with molds (Espinel-Ingroff, 2001).

Systemic mycoses that mostly treated by AmB, including those which are cause by opportunistic fungi such as *Aspergillus* spp., *Candida* spp., and zygomycetes and those by primary pathogenic fungi such as *Histoplasma capsulatum*, *Blastomyces spp., Coccidioides immitis, Cryptococcus spp.* and *Paracoccidioides* spp. (Dupont, 2002; Adler-Moore and Proffitt, 2008; Torrado *et al*., 2008; Peçanha *et al*., 2016).

Intravenous injection of 3.39 mg/kg/day of ABLC in 23 patients with paracoccidioidomycosis revealed 100% curative rate (Peçanha *et al*., 2016). Treatment of mice infected with two strains of *Aspergillus fumigatus* (wild and azole resistance strains) by 16 mg/kg of L-AmB for 14 days exhibited 100% survival rate (Seyedmousavi *et al*., 2017). Additionally, antifungal activity of AmB can increase after combining with other immune materials such as IFN-γ which increased the effectiveness of AmB against *A. fumigatus*, *Saccharomyces cerevisiae*, but not against *C. albicans* (El-Khoury *et al*., 2017). A pharmacokinetic/pharmacodynamic (PK/PD) model which is *in vitro* designed to simulate release of AmB from plasma against three clinical isolates of *Aspergillus* spp. shown that *A. fumigatus* was completely inhibited at C_{max} of ≥ 2.4 mg/liter and partial inhibition of *A. flavus* with growth delay

of 1 to 50 hours at C_{max} of 0.6 to 4.8 mg/liter, while it delays *A. terreus* growth over 8 hours for all C_{max}s. (Al-Saigh *et al*., 2013). Leishmaniasis is also can treat by AmB in which 85% of cutaneous leishmaniasis and 77% of old world mucosal leishmaniasis caused by *Leishmania infantum* was healing after treated with AmB (Mosimann *et al*., 2018).

Nanotechnology approach is a promising field to increase the antifungal activity of AmB through increasing deposition of it in spleen and liver, but not in kidney or lung and also to decrease its adverse effects on the human body (Torrado *et al*., 2008). Nanoemulsions containing AmB and cholesterol had been shown higher curative rate against Leishmaniasis with limited toxicity toward macrophages (Caldeira *et al*., 2015).

2. 9. Amphotericin B for treatment of animal model:

In vitro test of any new drug is always considered the first step to evaluation its therapeutic activity, followed by chosen a suitable animal model to determine the therapeutically nature of such new drug (Ishii *et al*., 2017). Usage of animal model, which is used as alternative for human infection, provides the answer for many questions about the mechanism of pathogenesis and host defense against infection (Shimamura *et al*., 2012). For dermatophytes infection, animal model introduces many benefits to understand dermatophytes pathogenesis, evaluation of new drug activity and increasing our knowledge of immune response mechanisms (Cambier *et al*., 2017). Otherwise, a variation between human and animals in the immune response, causative fungal agents, and difference in skin structure make a challenge in the establishment of dermatophytic infections in animal model (Shimamura *et al*., 2012). However, other difficulties in using of animal model to evaluate new drugs of dermatophytoses may include low response of

rodents to anthropophilic dermatophytic infections, inflammation results from preparation of infection site by shaving, and chosen of suitable animal model (Cambier *et al*., 2017).

A dog is chosen to be a model to evaluate curative ability of free polyaggregated amphotericin B (FPA) against infection by *Leishmania infantum*. No significant enhancement in clinical or parasitological characters was found after 6 months of intravenous injection of FPA (5 mg/kg) for three times every two weeks (Hernández *et al*., 2015). Experimental infected of mice with *Leishmania major* is used to evaluate the efficiency of therapeutic combination of AmB and chitosan platelets against such type of parasitic infection. Histological and immunohistochemical examination of treated skin lesion revealed a significant reduction of inflammatory granuloma and parasite load compared with D-AmB alone (Malli *et al*., 2019). Another combination of AmB 3% and oleic acid 5% in emulgel formula also showed the same efficiency in the treatment of cutaneous leishmaniasis in mice model after using in twice a day for twelve days (Pinheiro *et al*., 2016).

The application of AmB or any of other new drugs as suitable therapeutic chose of dermatophytosis in animal model will achieve the purpose to develop a new drugs used by the human (Cambier *et al*., 2017).

2. 10. Topical usage of Amphotericin B:

Intravenous administration is the common clinical usage of AmB to treat various systemic fungal infections in the human body (Torrado *et al*., 2008; Peçanha *et al*., 2016). Meanwhile, topical application of AmB is not commonly used and still under primary research. Compresses soaked in a solution of 5 mg ABLC was successfully used every 2 days until 5 weeks as topical treatment during postoperative period of patient with rhinomaxillary mucormycosis caused by *Lichtheimia ramose* (Trasmonte *et al*., 2012). Bronchial instillation is another type of tropical used of AmB. A patient with pulmonary chromomycosis caused by *Scedosporium prolificans* that developed after lung transplantation failed to response to systemic itraconazole, while improvement of bronchial obstruction was noticed after 3 instillations by AmB which was continuous as once every 3 months for 2 years (Mitomo *et al*., 2018).

A gel of AmB and γ- cyclodextrin complex was *in vitro* tested against 11 different fungal species and *in vitro* and *in vivo* against Leishmaniasis and its causative agent. An antifungal efficiency was observed with 48%, 28%, and 69% higher compared with AmB Neo-Sensitabs® disks, AmB dissolved in dimethyl sulfoxide and clotrimazole cream, respectively. The complex also revealed high *in vitro* leishmanicidal efficiency and *in vivo* activity against an experimental model of cutaneous Leishmaniasis (Ruiz *et al*., 2014). A structure of ultradeformable liposomes containing AmB (AmBUDL) with 107 ± 8 nm diameters is prepared to test antifungal activity of AmB and its characters on mammalian skin cells. It's revealed a significant antifungal activity against *C. albicans* and non-albicans Candida with less cytotoxic effects on mammal cells and 40 times higher accumulation rate on the human

skin than AmBisome. It also displays 100% of *L. brasiliensis* promastigot and 75% of amastigote at 1.25 µg/ml (Perez *et al*., 2016). Moreover, vaginal suppositories of 50 mg AmB were showed a successful treatment of 70% of ten women with non-albicans Candida vaginitis after given nightly for 14 days. The suppositories medicine is also revealed less local side effects and well tolerated (Phillips, 2005).

Preparation of topical eye drop of AmB is progressively developed. Liposomal AmB (AmBisome®) as 0.5% (w/v) eye drop is suggested to be alternative chose to Fungizone® of 0.15% (w/v) D-AmB which cause irritant for cornea. The stability of L-AmB new drop is quite good for 6 months at room temperature or at temperature from +2 to -8 °C (Morand *et al*., 2007). Topical AmB as eye drop may fail to cure fungal eye infection in some cases. Thus, a combination of AmB with other antifungal agent could increase its therapeutic activity. Topical voriconazole and AmB eye drop for 6 weeks revealed a successful treated activity of women with keratitis caused by *Scedosporium apiospermum* after AmB failed alone to give a positive curative result (Fadzillah *et al*., 2013).

Treatment with topical AmB may not always give high efficiency than when it given as ordinary form in treatment of fungal infection. Therapeutic efficiency of topical L-AmB solution in the treatment of 110 patients with cutaneous leishmaniasis for 8 weeks shown no significant different from that of intralesional glucantime (Layegh *et al*., 2011). Incorporation of AmB in nanoparticles is a new approach to use AmB as tropical drug for treatment of fungal infection. Nanoparticles encapsulated AmB (AmB-np) exhibited a significant *in vitro* and *in vivo* inhibitory activity against *Candida* spp. Growth and biofilm metabolic activity of *Candida* spp. is reduced to 72.4-91.1% and

80%-90%, respectively after 4 hours of *in vitro* tested. By using a murine full-thickness burn model, topical AmB-np showed a quicker efficiency to treat wound of mouse infected with *Candida* spp. during three days (Sanchez *et al*., 2014). Theoretical design of nanoemulsion formula of AmB based on pseudo-ternary phase diagram is also developed to recommend usage of AmB as topical treatment of skin infected with candidiasis and aspergillosis and to reduce its side effects (Sosa *et al*., 2017). A stable formula of AmB in microtube nonmaterial of 12-hydroxystearic acid (1%) had shown a similar antifungal efficiency of D-AmB against skin pathogenic fungi (Salerno *et al*., 2013). Solid lipid nanoparticles (SLNs) are another carrier design of vehicle containing AmB to increase its topical antifungal activity. This formula exhibited a high drug skin permeation and more inhibitory action against *Trichophyton rubrum* (Butani *et al*., 2016).

Chapter Three

Materials and Methods

3. Materials and Methods

3.1 Materials:

3.1.1 Apparatuses and Equipment

All Apparatuses and instruments used in this study were illustrated in table 1 and table 2.

Table (1): Apparatuses that used in the current study:

NO.	Apparatuses	Company	Country
1	Autoclave	Hirayama	Japan
2	Centrifuge	Hettich	Germany
3	Compound microscope	Leica	
4	Digital balance	Kern	
5	Bunsen Burner	Jenway	
6	Different size of Micropipettes	Slamed	
7	Biological safety cabinet	Lab Tech	Korea
8	Incubator	Fisher scientific	

9	Oven	Fisher scientific	Germany
10	Centrifuge	Hettich	
11	Water bath	GFL	
12	Vortex	Germany	USA
13	Refrigerator	Vestal	Turkey

Table (2): Equipments used in this study

NO.	Equipments	Company	Country
1	Gloves	Salalah medical supplies	Oman
2	Glass slide	Supertek	India
3	Funnel 500 ml	Marienfeld	Germany
4	inoculating loop	Loop shandon	England
5	Microscope cover slide	Supertek	India
6	Pyrex beakers	Marienfeld	Germany
7	Pyrex cylinder		
8	Pyrex Conical flask		
9	Petri dish	plastLab	Lebanon
10	Scalpel	Zepf medical instruments	Germany
11	Shaving machine	alpapazarlama	Turkish
12	Cotton	Wessam	Egypt

3.1.2 Chemical and biological materials

All of chemical and biological materials used in this study were mentioned in table 3.

Table (3): Chemical and biological materials

No.	Chemical and biological materials	Company	Country
1	Deoxycholate amphotericin B	Cipla	India
2	Clotrimazole cream (Fungicin® 1%)	HYAT drug producing Co.	Iraq
3	Solid basic cream	Wadi AlRafidain	Iraq

3.2. Methods:

3.2.1 Media prepared in the laboratory

3.2.1.1 Sabouraud's glucose agar (SGA)

Sabouraud's glucose agar (SGA) media was prepared by dissolving 20 g glucose; 15 g agar and 10 g peptone in a conical flask containing 950 ml of distill water. They were shaking together until complete dissolved. Final volume of solution was completed to one liter of water. Components were sterilized by autoclave for 20 minutes at 121 °C/1 pound. After cooling to 45 °C, chloramphenicol (0.05 g/l) was added into media to prevent growth of

bacteria. Sterilized media was poured in Petri dish and kept in refrigerator at 4°C until use (AL-Janabi, 2011).

3.2.2 Preparation of Amphotericin B cream 1%

Amphotericin B cream was prepared at concentration 1% by very well mixing of 10 mg of crystal powder of AmB in one gram of solid basic cream (Mithal and Saha, 2000). Approximately 200 g of AmB cream 1% was prepared and distributed in sterilized plastic cups. Each cup (20 ml) was contained 5 g of prepared cream to use in the treatment of infected animals and humans with dermatophytosis.

3.2.3 Treatment of Patients with AmB cream 1%

3.2.3.1 Patients

From 26 patients, a total of 12 volunteer patients with different type of dermatophytosis were continued involving in this study during attended to Al-Imam Al-Hussein Medical city in Karbala province of Iraq from October 2018 to July 2019. Gender of involved patients distributed between 10 males at age range 20-50 years and two females at age 18-52 years. Ethical approved was obtained from the committee of college of medicine, University of Karbala at 78 in 21 March 2018. Dermatophytosis was clinically diagnosis by specialized dermatologists of the hospital consultation department. Causative agents of dermatophytosis were determined by collecting skin specimens from the edge of the infected lesions after cleaning with 70% ethyl alcohol. Each skin sample was directly examined by the microscopic for detecting of any fungal elements, while some of them were cultured on SGA and incubate at 28-30 °C for 1-2 weeks. Grown fungi were examined microscopically. Fungal species was diagnosed based on the morphological characters of grown fungi and its

conidia, in addition to other observed characters such as colonies texture, color of front, reverse side of colonies and growth rate were also determined (Rippon 1988; Emmons *et al*., 1970).

3.2.3.2 Treatment of patients with AmB cream 1%

A total number of involved patients (12) was divided into two groups. First one included 6 patients were treated with AmB cream 1%, while second group of 6 patients was treated with clotrimazole cream 1%. Drugs was advice to use twice daily on the lesion area. Curative time was followed up for all treatment groups every week for determining the therapeutic activity of antifungal drugs and for monitoring any side effects. Clinical features of dermatophytosis lesions were determined and became under observation during study time. Severity of clinical features was designed to involve negative (-), mild (+), moderate (++), and sever (+++). Questionnaire was performed to all involved patients, including age, gender, occupation, site of infection on body, duration of disease, family history and any contact with animal.

3.2.3.3 Excluded patients

Several patients were excluded from this study, including pregnant women, elderly and children less than 16 years old, persons with known amphotericin B hypersensitivity, breast feeding mothers, and patients under any systemic antifungal therapy within two weeks or topical antifungal therapy within one week prior to study.

3.2.4 Treatment of infected animals

3.2.4.1 Preparation of fungal inoculum for animal infection

An isolated strain of *Trichophyton mentagrophytes* was obtained from male (37 years) with tinea corporis in his forearm admitted into the AI-Ammam AI-Hussein medical city hospital in October 2018. Skin scales were collected from infected lesion by scalpel and cultured on SGA after obtained a positive result of direct microscopic examination. Inoculated media was incubated at 28-30 °C for 1-2 weeks. *T. mentagrophytes* was diagnosed depending on morphological characters of conidia (macroconidia and macroconidia), hyphae, and other observed colony characters (Rippon 1988; Emmons *et al*., 1970). Suspension from fungal culture was prepared by mixing a few colony parts of it with 100 ml of sterilized distil water in conical flask (250 ml). Fungal suspension was used immediately after preparation.

3.2.4.2 Preparation of animals for treatment

Rabbit was chosen to be an animal model for treatment by new preparation of AmB cream 1%. A total of 12 healthy rabbits at weight 2.5 to 3.5 kg were used in this study. All of these rabbits were prepared to infect by *T. mentagrophytes*. About 5-7 cm of the neck area of each rabbit was shaved by mechanical machine to remove covering hairs and to clarify the skin layer. A few drops of prepared fungal suspension were inoculated on the shaved area with some pressured and spreading by hand fingers. Infection development was follow up for more than 3 weeks. Lesion was clinically evaluated as dermatophytosis infection after inoculating periods.

3.2.4.3 Treatment of animals

All of infected rabbits were divided into four groups with 3 rabbits in each. First group was treated with prepared AmB cream 1%. Second group was treated with clotrimazole cream 1%. Third group was treated with cream only. Forth group was left as control without treatment. First three groups were topical treated twice daily. Curative rate was followed up during the study time. Complete healing of infected rabbits was evaluated by totally disappear of clinical features of infected area. Meanwhile, a positive result of healing was confirmed by culturing a skin specimen from curative area on SGA in order to be sure for the absence of any remaining fungal elements. Rabbits were left under observation for months to record any recurrence infection after the end of the treatment.

3.2.5 Statistical analysis

The data of all tests were expressed as mean ± SD. The values were analyzed statistically with one way ANOVA by using windows Excel application version 10. The minimum level of p value was < 0.05 concerts as significant level.

Chapter Four

Results

4. Results:

4.1. Treatment of animals with dermatophytosis by AmB cream 1%

A total of twelve healthy rabbits were used in the current study as animal model for investigating of antifungal action of topical AmB for treatment of experimental dermatophytoses. Clinical features of dermatophytosis were observed on all animals after 7-14 days of infection by *T. mentagrophytes*.

The curative time of infected animal was found variable among different groups. After four days of treatment, animals of the first group that treated with AmB cream 1% showed a curative features with a significant difference ($p < 0.05$) from other groups, while a completing healing from infection was after six days (Fig. 1). The second group of involved animals that treated with 1% clotrimazole was started to cure after 14 days and completing healing after 28 days with a significant difference ($p< 0.05$) from two control groups (Fig. 2). Third and fourth groups of animals which were treated with a cream only or without treatment showed no depletion of infectious lesions for all of study periods (more than 90 days) (table 4). Moreover, dermatophytosis lesions did not spread from the origin site in animals of first and second group. Whereas, other two groups, especially those treated by cream only, revealed a distributed of lesions to a wide area of the body of some rabbits (Fig. 3)

The rabbits of first and second groups were kept under observation for three months to determine any possible recurrent infection after complete healing. Recurrence infection appeared in one rabbit treated with clotrimazole cream 1% after one week from completed healing, while none of the rabbits

treated with topical Amphotericin B showed like this recurrence for more than 4 months.

After completing of curative time, skin and hairs were scraped from treated area of the rabbits of group one and two. Examination of these samples was performed to insure about the complete removal or elimination of fungal cells. Negative results were obtained after direct microscopic examination and culturing of collected skin samples.

Table 4: Curative time of infected animals

Animal group	Treatment type	Curative time (days)
1	AmB cream 1%	4-6*
2	Clotrimazole cream 1%	14-28**
3	Cream only	> 90
4	Without treatment	> 90

*: significant differences between AmB group and other groups at $p < 0.05$

**: significant differences between clotrimazole group and controls at $p < 0.05$

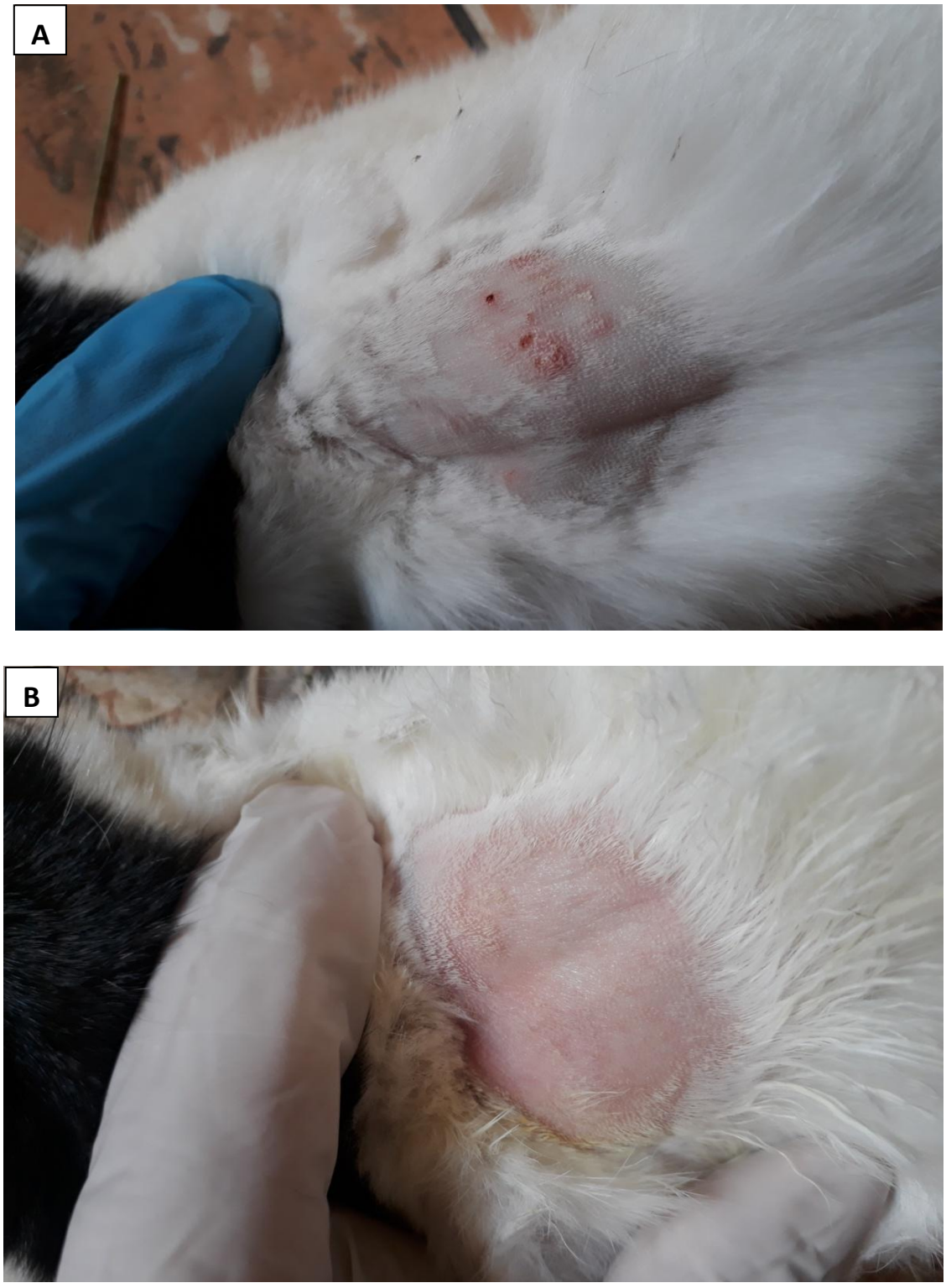

Figure 1: Rabbit treated with AmB cream 1% after 6 days

A: before treatment; B: after treatment

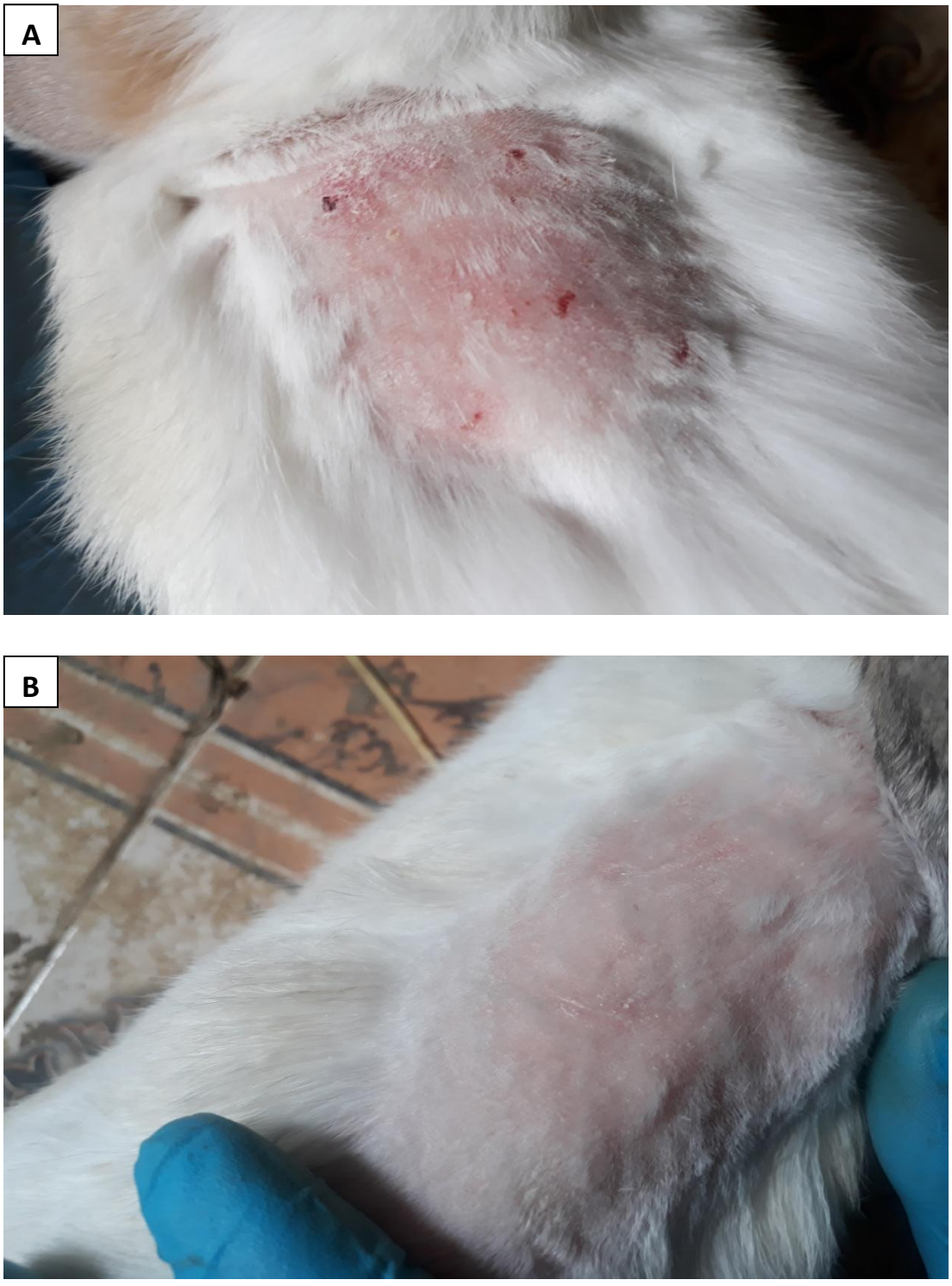

Figure 2: Rabbit treated with clotrimazole cream 1% after 28 days

A: before treatment; B: after treatment

Figure 3: Wide distribution of infection in rabbit treated with cream only

4.2. Gender and age of patients treated with AmB and clotrimazole creams:

Males were represented the greater number of patients with dermatophytosis involved in this study. In the first group who treated with AmB cream, there were 10 males and 2 females. The age of males with tinea corporis who treated with AmB cream were ranged between 20 and 50 years and 20 years for those with tinea barbae. Meanwhile, females with tinea corporis were at age range of 18-52 years. Otherwise, all of treated groups with clotrimazole cream were males at age range from 20 to 40 years (table 5).

Table 5: Age and type of dermatophytosis of treated patients

Treatment type	Gender	Patient age (years)	Type of tinea	No. of patients
AmB cream	Male	35 ± 12.2	Tinea corporis	3
		20 ± 0	Tinea barbae	1
	Female	35 ± 14.6	Tinea corporis	2
Clotrimazole cream	Male	30 ± 8.6	Tinea corporis	6
Total No.				12

Mean ± Standard deviation

4.3. Treatment of patients with dermatophytoses by AmB cream 1%:

The successful treatment of rabbits with topical amphotericin B cream in a short duration time and high efficiency encourages us to start the next step of treatment of infected human with dermatophytosis. Two groups with 6 dermatophytosis patients in each were involved in this study to determine the therapeutic efficiency of AmB cream 1%compared to clotrimazole cream 1%.

Patients of group one who treated with AmB cream 1% were cured in short time (10 days) for both types of tinea (tinea corporis and tinea barbae)(Figure 4). Meanwhile, group two who treated with clotrimazole cream needed more time (14-21days) to cure of all patients with tinea corporis (table 6).

Table 6: Curative time of patients with dermatophytoses

Patients group	Treatment type	Type of tinea	No. of patients	Curative time (days)
1	AmB cream 1%	Tinea corporis	5(41.66%)	10*
		Tinea barbae	1(8.33%)	10*
2	Clotrimazole cream 1%	Tinea corporis	6 (50%)	14-21
Total No.			12	

*: significant differences between AmB group and clotrimazole group at $p < 0.05$

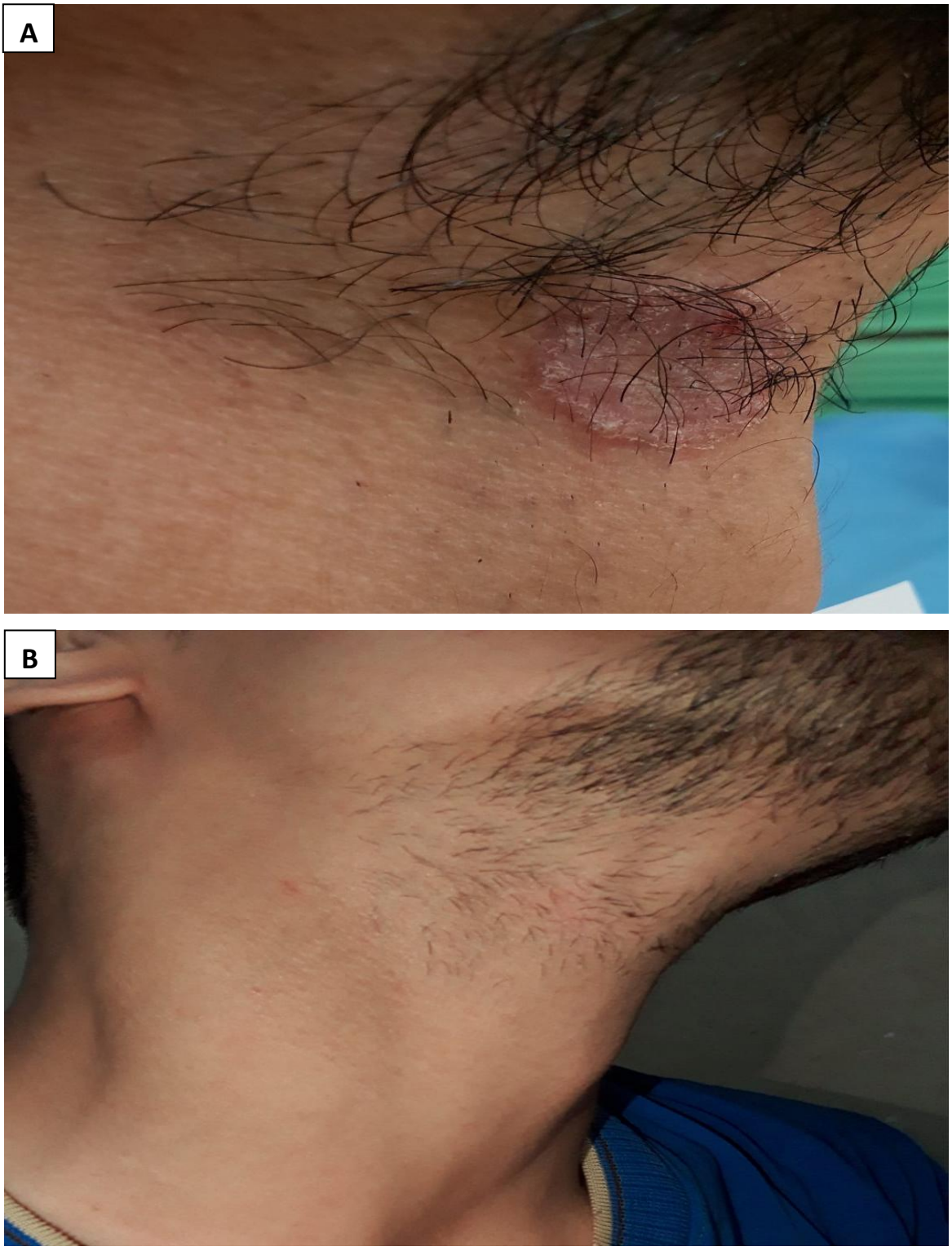

Figure 4: Patient treated with AmB cream 1%

A: before treatment; B: after 10 days treatment

4.4. Clinical features of treated patients:

Clinical features of patients treated with AmB cream showed variable degree in their intensity. Pruritus was observed in all involved patients, except one male with tinea corporis. All patients with the presence of pruritus were had a moderate degree, except one male and female with tinea corporis. Pain was not observed in all of involved patients. Two males with tinea corporis showed mild degree of scaling in lesion area and one with sever degree, while a moderate degree of scaling was observed in male with tinea barbae. Meanwhile, two infected females with tinea corporis showed a moderate degree of scaling (table 7).

Redness was found in all males and females who participating in this study. In males, redness was ranged from mild to moderate, while in females moderate to severe redness have been observed (table 7).

Vesiculation degree from mild to moderate was appeared in all male patients, except in one with tinea corporis. Whereas, females were had a moderate vesiculation degree in lesions area. In general, this study showed that females had more severity of clinical features associated with dermatophytoses than in males (table 7).

Table 7: Clinical features of treated patients with AmB cream 1%

No.	Gender	Type of tinea	Clinical features				
			Pruritus	Scaling	Pain	Redness	Vesiculation
1	Male	Tinea corporis	+++	+	-	+	+
2		Tinea corporis	++	+	-	+	-
3		Tinea barbae	++	++	-	++	+
4		Tinea corporis	-	+++	-	++	++
5	Female	Tinea corporis	++	++	-	++	++
6		Tinea corporis	+++	++	-	+++	++

(-) None; Mild (+); moderate (++), sever (+++)

4.5. Determination of the history of patients treated with AmB cream 1%:

History information of the patients treated with AmB cream was collected. There was no medical and drug history for all of patients. The duration of dermatophytoses development was ranged from one week to one month for tinea corporis, while it developed during 10 days for male with tinea barbae (table 8).

Occupation type was variable for males and females. Two males and one female were working as a student, while other males working as grosser and soldier and one female as wife house. Two females and one male with tinea corporis were in contact with birds which are mostly chicken, while a male with tinea barbae was in contact with a cat. Other two males with tinea corporis were had no history of animals contact (table 8).

Table 8: History of patient treated with AmB cream 1%

Gender	Type of tinea	Duration of disease	Occupation	Animal contact	Drug history	Medical history
Male	Tinea corporis	2 weeks	Soldier	-	-	-
	Tinea corporis	2 weeks	Grosser	-	-	-
	Tinea barbae	10 days	Student	Cat	-	-
	Tinea corporis	1 week	Student	Birds	-	-
Female	Tinea Corporis	2 weeks	Student	Birds	-	-
	Tinea corporis	1 month	House wife	Sheep & birds	-	-

4.6. Determination of dermatophyte types in patients treated with AmB 1%:

Culture of specimens scraped from dermatophytosis lesions showed the presence of two species of dermatophytes, *T. mentagrophytes* and *Microsporum canis.* The most common isolate of dermatophytes from tinea corporis lesion was *M. canis* (83.32%), while *T. mentagrophytes* was isolated from one patient (16.66%). Meanwhile, tinea barbae was found to be caused by *M. canis* (16.66%) (table 9).

Table 9: Type of dermatophytes isolated from dermatophytoses patients treated with AmB 1%

Type of disease	Patients No.	Type of dermatophytes
Tinea corporis	1(16.66%)	*T. mentagrophytes*
Tinea corporis	4 (66.66%)	*M. canis*
Tinea barbae	1(16.66%)	*M. canis*
Total No.	6	

Chapter Five

Discussion

5. Discussion:

5.1. Preparation of Amphotericin B (AmB) cream:

Amphotericin B (AmB) is the most common type of antifungal drugs used for treatment of various fungal infections in the human body (Baginski and Czub, 2009; Volmer *et al*., 2010). Its mechanism of antifungal action representing by pore forming in the fungal plasma membrane leading to cell death (Hartsel *et al*., 1993; Shimizu *et al*., 2010; Mesa-Arango *et al*., 2012; Gray *et al*., 2012; Stone *et al*., 2016). In addition to old formula of D-AmB, three lipid formulas were development in order to limit the adverse effects of D-AmB in the human body and increase its therapeutic activity (Dupont, 2002; Herbrecht *et al*., 2003; Hamill, 2013; Stone *et al*., 2016; Steimbach *et al*., 2017; Serrano and Lalatsa, 2017). All of known available formulas of AmB are administrated via intravenous injection (Torrado *et al*., 2008; Peçanha *et al*., 2016; Serrano and Lalatsa, 2017), while development of topical formula of AmB is still under preliminary development. These experiments focused on preparing topical drugs of AmB with different pharmaceutical forms such as eye drops (Morand *et al*., 2007; Fadzillah *et al*., 2013), gel (Ruiz *et al*., 2014), vaginal suppositories (Phillips, 2005), solution (Trasmonte *et al*., 2012) and bronchial instillation (Mitomo *et al*., 2018). In this study, a new formula of AmB cream 1% was prepared and evaluated as a topical treatment of dermatophytosis in animals and the human.

5. 2. Treatment of animals with dermatophytosis by AmB cream 1%:

Dermatophytosis can be developed in both of human and animals with some differences in clinical features (Radentz, 1991; Seker and Dogan, 2011; Moretti *et al*., 2013; Pin, 2017). Zoophilic group of dermatophytes is the most causative agents of dermatophytosis in the human and animals (Radentz, 1991). Development of dermatophytosis on the animal body may required one week, while clinical signs need 2-4 weeks as shown by infected the animal with *M. canis* (Spickler, 2013). The human can easily get dermatophytes agents from contact with different types of animals such as cats, dogs, Guinea pigs and rabbits (Havlickova *et al*., 2008). Thus, choosing of animal as a model to infect with dermatophytes will elevate the successful rate of the process to develop a new drugs and also to prevent used of the human as experiment model (Scorzoni *et al*., 2017). Scientific ethics and safety requirements are preventing usage of the human as preliminary experimental subjects to evaluate the new drug or to determine the pathogenesis of any disease as with dermatophytosis (Shimamura *et al*., 2012).

Rabbits were used in this study as animal model for evaluating the therapeutic activity of new formula of AmB against dermatophytic infection. This choosing was depended on the fact that rabbits are porn to infect with cutaneous fungal infection without significant affected by the age, sex, and body weight of the rabbits (Dey *et al.*, 2016).

Trichophyton mentagrophytes was selected to be an infectious agent in our rabbit group for development of dermatophytosis. This fungal species found to be more causative agent of dermatophytosis in rabbits (Krämer *et al.*, 2012; Kraemer *et al*., 2012; Dey *et al.*, 2016). Incidence of dermatophytosis caused

by *T. mentagrophytes* var. *granulosum* was found higher in farm rabbits (79.5% from 220 farm rabbits) (Torres-Rodriguez *et al*., 1992; Moretti *et al*., 2013). Otherwise, Adult healthy rabbits may consider a carrier to dermatophytes without showing any signs of infection (Moretti *et al*., 2013). However, other species of dermatophytes, such as *Microsporum canis* or *Microsporum gypseum* can also be causing dermatophytosis in rabbits, especially pet rabbits (White *et al*., 2003).

In this current study, application of AmB cream 1% for treatment of dermatophytosis in animals was investigated. The AmB cream showed more effective action against experimental dermatophytosis in rabbits with a short time of curing in compared to clotrimazole cream or control group. Dermatophytosis lesions were completely cured after 4-6 days of treatment with AmB cream compared with 14-28 days of clotrimazole cream. This time of curing also noticed when infected rabbits with dermatophytosis topically treated with aqueous garlic extract twice daily for 14-17 days (Amer *et al*., 1980).

Topical application of AmB and γ- cyclodextrin gel complex for treatment of cutaneous leishmaniasis in animal model showed more curative rate than clotrimazole cream (Ruiz *et al*., 2014). An emulgel formula of AmB 3% and oleic acid 5% also revealed effective results for the treatment of cutaneous leishmaniasis in mice model after using it twice a day for twelve days (Pinheiro *et al*., 2016). Moreover, mouse infected with *Candida* spp. was also successfully treated with topical AmB nanoparticles after 3 days as mentioned by the results of murine full-thickness burn model (Sanchez *et al*., 2014).

All of treated rabbits with new AmB cream did not show any recurrence infection for more than 4 months after completing the curative time, while the dermatophytosis infection was recurred in one rabbit of treated group with clotrimazole cream 1%. However, recurrence of dermatophytosis is the most serious problem should be considered after completing the curative time (within 4 weeks of stopping therapy) (Gupta *et al*., 2008; Sinha and Sardana, 2018). It could be resulted from return contact with infection source or from failed of treatment with antifungal drug (Dogra and Uprety, 2016). Dermatophytosis recurrence is found commonly among Indian patients with tinea faciei (100%), tinea pedis (80%) and tinea unguium (46.6%) in which its rate was 34.3% after treatment (Sharma *et al*., 2017). Whereas, it found more frequent in those with tinea cruris and tinea corporis in another part of India (Ranganathan *et al*., 1995). Recurrence in cases of vaginitis infection by non-albicans Candida was diagnosed in women after failed treatment with AmB (Phillips, 2005). Dermatophytosis relapse is also detected in patients after 1-4 weeks of treatment with clotrimazole cream (Bergstresser *et al*., 1993). However, factors such as depth of infectious lesions and even socio-economic conditions may be responsible for the recurrence of dermatophytosis infection (Van Cutsem and Janssen, 1984; Ranganathan *et al*., 1995)

5. 3. Treatment of patients with dermatophytoses by AmB cream 1%:

Several different drugs are used today for topical treatment of dermatophytosis infection. Itarconazole of azoles group and terbinafine of allylamines group are the most common type of topical treatment of dermatophytosis (Gupta *et al*., 2008). Long duration periods of treatment, drug resistance and even the cost are the most problems associated with the usage of known antifungal agents (Scorzoni *et al*., 2017). Thus, discover of new antifungal agent will take the priority for enhancement treatment of various fungal infection, including dermatophytosis.

Dermatophytosis is usually need at least 2-4 weeks to be cured in approximately all of its types and may reach to six months in cases of tinea capitis and onychomycosis (Gupta *et al*., 2008; Al-Janabi, 2014; Hay, 2018). Our current preparation of AmB cream revealed a significant therapeutic activity for the treatment of dermatophytosis in the human during 10 days compared to clotrimazole cream. As with other topical drugs, treatment of our involved patients with tinea corporis and tinea barbae advise to be applied twice daily. This process of topical treatment, once or twice daily, is mostly required for treatment major types of tinea (Gupta *et al*., 2008). Although clotrimazole has more antifungal activity against dermatophytes when it tested *in vitro* (Fernández-Torres *et al*., 2001), clotrimazole cream used in this study showed less therapeutic action against dermatophytosis than AmB cream. Actually, there are always differences between the results of *in vitro* and *in vivo* exterminates. These differences may be related to either of the host conditions, such as immune response, site of infection, and underlying illness or to the fungal characters as with virulence, or to the antifungal agent, such as

dose, pharmacodynamic, pharmacokinetics and drug interaction (Sinha and Sardana, 2018).

There are many advantages from using of AmB as topical treatment of dermatophytosis. Firstly, discover a new drugs or modification old one will participate to increase the available limited number of antifungal drugs (Scorzoni *et al*., 2017). Secondly, topical preparations are much less costly than orally administered antifungal drugs and cause minimal adverse side effects (Crawford and Hollis, 2007; Hay, 2018). Third, the application of topical formula of AmB considers more safety to use and will not produce clinical relevant serum levels of AmB (Pendleton and Holmes, 2010; Hay, 2018). Forth, the failure of other antifungal agents to treatment cutaneous fungal infection will be resolved as noticed when used topical AmB (0.1% w/w) against sporotrichosis (Mahajan *et al*., 2015). Fifth, quality of patient life will increase if new drug improved to cure infectious lesions in short time (Scorzoni *et al*., 2017).

5.4. Gender and age of patients treated with AmB cream 1%:

Dermatophytosis can be development in both of males and females with some differences in prevalence rate between various geographical areas. Generally, there is no sexual predilection for dermatophytosis infection (Al-Janabi, 2014). Males with dermatophytosis were represented the great number of involved patients in this study. Superficial mycoses found more common in Nepal males (77.5%) than in females (22.5%) (Khadka *el al*., 2016). From 34 positive cases of dermatophytosis in Baghdad province, male patients (62%) were more frequently infected than females (38%) (Mohammed *et al*., 2015). Males were also revealed higher rate of dermatophytosis infection when they represented 64.8% compared to 35.2% females from 88 positive patients with

dermatophytosis in West of Iran (Farokhipor *et al*., 2018). On the other hand, other studies recorded that females had more frequently infection rate with dermatophytosis than males (Teklebirhan and Bitew, 2015; Dogo *et al*., 2016).

The age of involved patients in this study was ranged from 20-50 years for males and 18-52 years for females. The peak of dermatophytosis incidence is usually higher after puberty (Al-Janabi, 2014; Poluri *et al*., 2015). Children below 10 years are also susceptible to infection with dermatophytosis (Mohammed *et al*., 2015). Ethiopian population found to be more frequently infected with dermatophytosis at age 25-44 years and at 45-64 years (Teklebirhan and Bitew, 2015), while other study recorded that Ethiopian population more infected at 1-14 years and at 25-44 years (Bitew, 2018). However, differences in age may not show significantly effects on the prevalence of dermatophytosis. The prevalence of tinea capitis in the Nigeria children was lower in age group of 5-10 years than for 11-15 years, but without significant differences (Dogo *et al*., 2016).

5.5. Clinical features of treated patients:

Dermatophytosis or tinea is usually presented with variable clinical features depending on the location of infection, type of dermatophytes and the immune state of the host (Tainwala and Sharma, 2011). General features of tinea on infected skin of the human represented by the presence of an annular patch with an advancing, raised, scaling border and central clearing (Andrews and Burns, 2008; Spickler, 2013). These features may show variable degree of scaling and inflammation reaction which could extension to form scaring and alopecia area (Tampieri, 2004). Thus, inflammation and erythrermatous signs are clearly identified in severe type of dermatophytosis infection (Al-Janabi, 2014). Other clinical features such as itching, maceration, pain, scaling,

vesicles or plaster forming, and erthrymatous rate are variable between mild to modern degree (Andrews and Burns, 2008; Moriarty *et al*., 2012; Al-Janabi, 2014). The trigger to develop such clinical signs is mainly by diffusible of fungal metabolites through the malphigian layer of skin and induces host response (Tainwala and Sharma, 2011).

From this study, variable clinical signs were recorded among involved patients with dermatophytosis who treated with AmB cream, including pruritus, scaling, pain and vesicles. Tinea corporis and tinea cruris are the most type of tinea represented with pruritic and erythrematous rash lesions and containing of pustules or vesicles with an active scaly palpable edge (Moriarty *et al*., 2012). However, these features are mostly associated with tinea cruris (Andrews and Burns, 2008) and less common in tinea corporis (Al-Janabi, 2014). Itching is an addition sign of dermatophytosis infection which depends on the site of infection and it usually appeared very mild in case of tinea corporis and very intense in tinea cruris (Al-Janabi, 2014). Tinea pedis which is located between the fingers of the foot is characterized by the presence of scaling, maceration and less commonly with small vesicles and blisters (Moriarty *et al*., 2012; Al-Janabi, 2014). However, clinical signs such as erythrema, itching, scaling, margins and size of lesion can be used as indicator to determine the therapeutic ability of some antifungal drugs (ShivaMuRthy *et al*., 2014).

5. 6. Determination the history of patients treated with AmB cream 1%:

Dermatophytosis is often initiated from the contact of viable fungal arthrospores or hyphae with the skin surface of the human which later encourage to adhesion and germinate to form infection by the presence of suitable conditions (Tainwala and Sharma, 2011). Incubation period on the human skin for development of dermatophytosis is usually from 1 to 2 weeks (Spickler, 2013). Humidity and warm temperature are the most effective factors for infection development (Al-Janabi, 2014). However, dermatophytosis infection can be increased in the presence of several conditions such as overcrowding, dressing of occlusive cloths, increased urbanization, low socioeconomic status, contact with animals and poor hygiene (Torrado *et al*., 2008; Jaulim *et al*., 2015).

In the recent study, the duration time of tinea corporis was ranged from 2 weeks to one month, while it was 10 days for tinea barbae. Generally, superficial mycosis is usually shown a low tendency to self limitation (Torrado *et al*., 2008; Jaulim *et al*., 2015). For dermatophytosis, most of the healthy human or animals tend to be self-limiting within weeks to months (Spickler, 2013; Moriello *et al*., 2017). Treatment can short the course of the disease to prevent spread to other animals and peoples (Spickler, 2013; Jaulim *et al*., 2015; Moriello *et al*., 2017). Thus, poor medical care will increase the epidemic spread of skin mycoses (Torrado *et al*., 2008).

Most infected persons in this study were found to have a history to contact with animals and birds, which make us, suggest that the rout of transmission is mostly by contact with animals. Animals consider the main reservoir of

zoophilic dermatophytes (Moriarty *et al*., 2012; Mattei *et al*., 2014). These zoophilic and even geophilic dermatophytes can easy transmit to the human (Spickler, 2013). Thus, the human in contact with infected animals which may be pets, domestic, or wild animals is always at risk to get dermatophytic infection (Tampieri, 2004; Torrado *et al*., 2008; Spickler, 2013). The pet population has been increased in the last years due to raise interesting of people to have this small animal and spend most of time with them, especially children (Mattei *et al*., 2014). Three of 11 cases of children was acquired dermatophytosis from infected rabbits which used as pets by their family (Krämer *et al*., 2012). Thus, individual who is in contact with animals during his works as farmer or even when he works at home will be at risk to get dermatophytosis (Alzubaidy *et al*., 2018).

5. 7. Determination of dermatophyte types in patients treated with AmB cream 1%:

Tinea corporis can be the most diagnostic dermatophytosis type among our involved patients. This result also mentioned by other studies. From 115 patients with dermatophytosis in Baghdad, tinea corporis (26.7%) was the high prevalence than other types of tinea, while tinea manuum was less prevalence (Alzubaidy *et al*., 2018). This also recorded in India when tinea corporis (35.4%) represented the most predominant type followed by tinea cruris (16.8%) and tinea capitis (16.7%) (Balakumar *et al*., 2012).

Microsporum canis was considered the most frequently isolates from tinea corporis of our involved patients. It also found with higher prevalence (50.3%) than *T. mentagrophytes* var. *mentagrophytes* (35.4%) in patients of Southeast Serbia (Otasevic *et al*., 2011). Another study performed in the same

Southeast Serbia during six years (2012-2017) also confirmed that *M. canis* (63.9%) was the most prevalence than *T. mentagrophytes* (21.8%) (Otasevic *et al*., 2019). In Saudi Arabia, *M. canis* was most prevalence species of dermatophytosis and it represented 5.8% as causative agent of tinea corporis (Khaled *et al*., 2015). Generally, *Trichophyton* species are the most causative agents of dermatophytosis in the human, followed by *Microsporum* spp. and less by *Epidermophyton* spp. (Aditya *et al*., 2005; Khadka *et al*., 2016; Van *et al*., 2019). From *Trichophyton* genus, *T. rubrum* is the common isolate from dermatophytosis lesion, followed by *T. mentagrophytes* (Surendran *et al*., 2014; Bhagra *et al*., 2014; Jena *et al*., 2018; Kadhim, 2018; Adesiji *et al*., 2019). *T. mentagrophytes* could also be common in other studies as found when it isolated from 30 positive patients in Baghdad which represented 21.7% compared with *E. floccosum* (17.4%) and *Trichophyton bullosum* and *Trichophyton tonsurans* (13.0%) (Alzubaidy *et al*., 2018).

Conclusions and Recommendations

Conclusions:

1- Topical Amphotericin B cream 1% is effective drug for treatment of dermatophytosis in animal and human with significant differences from clotrimazole cream 1%.

2- Recurrent of infection is absence after treatment with topical Amphotericin B cream 1%.

3- *Microsporum canis* is more frequently cause of dermatophytosis in human.

4- Tinea corporis is more common type of dermatophytosis in human, especially in males.

5- Contact with animals is important risk factor to get dermatophytosis.

Recommendations:

1- Make other studies about the possible toxicity of AmB cream in animals are needed.
2- Determine the pharmaceutical characters of AmB cream 1% after treatment is required.
3- Determine the stability and absorption of AmB cream by the skin needs to determine.
4- More species of fungi required to test efficacy of AmB against them *in vivo.*

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الخلاصه:

لا يوجد في الحقيقة جزء من العالم خالي من الإصابة بمرض السعفة. يكون جلد وشعر وأظافر جميع أنواع الثدييات، بما في ذلك الإنسان، معرضون لخطر الإصابة بمرض السعفة. يتولد مرض السعفة بشكل رئيسي عن أنواع مختلفة من الفطريات الجلديه عند تواجدها داخل الطبقة الجلدية. تستخدم العديد من مختلف الادوية الموضعية والجهازية لمعالجة مرض السعفة. اذ يستخدم الأمفوتريسين ب عن طريق الحقن على نطاق واسع لعلاج الاصابات الفطرية الجهازية، اما الشكل الموضعي لدواء الامفوترسين ب فلا يزال تحت المستوى التجريبي.

تم تحضير شكل موضعي من الأمفوتريسين ب (% 1) لاستخدامه كعلاج لمرض السعفة في الحيوان والبشر وتم اختيار الأرانب لتكون نموذجًا حيوانيًا للعلاج بالمستحضر الجديد من كريم الامفوترسين ب، اذ تم إصابة 12 أرنب سليم بواسطة العزلة السريرية *Trichophyton mentagrophytes*. كما تم تقسيم الأرانب إلى أربع مجموعات تحوي كل مجموعة على 3 أرانب. تمت معالجة المجموعة الأولى مرتين يوميًا بكريم الأمفوتريسين ب 1٪ والمجموعة الثانية مع كريم كلوتريمازول 1٪ مرتين يوميًا والمجموعة الثالثة مع كريم فقط وتركت المجموعة الرابعة دون علاج كمجموعة سيطرة. اظهر الأمفوتريسين ب المعالج به الحيوانات فترة علاج أقصر (4-6 أيام) من كريم كلوترامازول (14-28 يومًا) مع وجود اختلافات معنوية ($P < 0.05$).

اشترك في البحث مجموعتان تمثل 12 مريضا يعانون من السعفة في هذه الدراسة. تم علاج المجموعة الأولى المؤلفة من ستة مرضى بكريم الأمفوتريسين ب، في حين عولج ستة مشاركين آخرين بواسطة كريم كلوتر امازول 1 ٪ وكما هو الحال مع الحيوانات المعالجة فان الفترة العلاجية بكريم الأمفوتريسين ب كانت أقصر (10 أيام) مقارنة مع كريم كلوتريمازول (14-21 يومًا) مع وجود اختلافات معنوية ($P<0.05$).

ان سعفة الجسم كانت الأكثر شيوعا" (91.66٪) عند المرضى المشتركين بالدراسة، تلتها سعفة اللحية (8.33٪)، وتم عزل الفطر *Microsporum canis* بشكل متكرر (83.32٪) من مرضى سعفة الجسم، بينما تم عزل الفطر *T. mentagrophytes* من مريض واحد (16.66٪)، وكان الذكور في الفئة العمرية (20-50 سنة) أكثر إصابة بسعفة الجلد من الإناث (18-52 سنة). تراوحت المظاهر السريرية لمرض السعفة ما بين الخفيفة إلى الشديدة بالنسبة لمعظم المرضى

المشاركين في هذه الدراسة، وكان نوع المهنة للذكور والإناث متغيرًا ما بين مهنة الطالب والمهن الاخرى، كما كان بعض المرضى المشاركين بتماس مع الحيوانات والطيور.

جمهورية العراق
وزاره التعليم العالي والبحث العلمي
جامعه كربلاء/ كليه الطب
فرع الاحياء المجهرية

العلاج الموضعي لمرض السعفة في الانسان بواسطه تركيبه جديده من الامفوتريسين ب باستخدام الحيوان كنموذج

رسالة مقدمة الى
مجلس كلية الطب جامعة كربلاء
كجزء من متطلبات نيل شهادة الماجستير في الاحياء المجهرية الطبية

من قبل
فلاح حسن عبيس الخيكاني
بكالوريوس الكليه التقنيه الطبيه بغداد (2013)

بأشراف
البروفسور علي عبد الحسين صادق الجنابي
د. لـــؤي محـــمد الربيعـــــي

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