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APPLICATION OF CHITOSAN IN THE CONTROL OF FUNGAL INFECTIONS BY DERMATOPHYTES

by

Ana Isabel Ribeiro Lopes

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APPLICATION OF CHITOSAN IN THE CONTROL OF FUNGAL INFECTIONS BY DERMATOPHYTES

Aplicação do quitosano no controlo de infecções fúngicas por dermatófitos

Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirements of Master of Science degree in
Applied Microbiology

by
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Place: Escola Superior de Biotecnologia of the Universidade Católica Portuguesa

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July 2013

To my mother and my father.

To my sisters Teresa and Inês.

Resumo

Os dermatófitos são um grupo de fungos que conseguem invadir as superfícies queratinizadas de humanos e outros animais e originar uma infeção - dermatofitose. Estas infeções são um importante problema de saúde pública e, para as controlar, é necessário terapia prolongada. No entanto, os medicamentos existentes parecem exibir efeitos secundários e o uso frequente e prolongado destes compostos é responsável pela existência de estirpes resistentes a antifúngicos, o que representa um risco potencial para o ambiente e saúde humana. Por isso, são necessárias novas drogas biocompatíveis para usos prolongados. O quitosano é um polissacarídeo catiónico e biocompatível que possui atividade antimicrobiana. Assim, o principal objetivo deste trabalho foi avaliar a atividade antifúngica do quitosano sobre alguns dermatófitos e algumas espécies de *Aspergillus* sp.

Para isso, a concentração mínima inibitória dos quitosanos foi determinada e os resultados mostraram que o quitosano possui atividade antifúngica contra *T. rubrum* e *M. canis*, apresentando CMIs que variam entre 1.1 e 2.2 mg/mL. Para as espécies de *Aspergillus*, não foi possível determinar as CMIs do quitosano. A concentração mínima fungicida também foi obtida para os dermatófitos, apresentando os mesmos valores obtidos para as CMIs.

Como os dermatófitos são responsáveis por infeções das superfícies queratinizadas, é preciso compreender se o quitosano exerce algum efeito na atividade fúngica. A análise de imagens de microscopia eletrónica de varimento mostou que o quitosano parece ter um efeito protetor do substrato usado, o cabelo, quando este foi infetado com dermatófitos.

O estudo do efeito do quitosano na atividade enzimática usando protease K revelou uma atividade importante na prevenção da ação das proteases. O efeito do quitosano na degradação de queratina por *M. canis* e *T. rubrum* também foi estudado pelo teste da "keratin-azure" e os resultados indicaram que a libertação de cor era menor quando o quitosano estava presente no meio de cultura.

A análise microscópica da superfície da "keratin-azure" quando o quitosano estava presente no meio de cultura corroborou as conclusões anteriores porque a superfície da "keratin-azure" tratada com quitosano mostrou-se intacta, apesar da existência de estruturas fúngicas à sua volta.

Com base nestes resultados, é possível concluir que o quitosano apresenta uma atividade antifúngica relevante contra os dermatófitos, mostrando-se uma alternativa promissora aos tratamentos comuns para a tinha.

Abstract

Dermatophytes are a goup of fungi that can invade keratinized tissues of humans and other animals and produce an infection - dermatophytosis. Dermatophytic infections are an important public health problem and to control them a prolonged therapy is required. But, the existing drugs seem to exhibit side effects and the frequent and prolonged use of these compounds is responsible for strain resistance, representing a potential risk for the environment and human health. Because of this features, new drug biocompatible formulations for long term use are required. Chitosan is a cationic, biocompatible polysaccharide that possess antimicrobial action. So, the main objective of this work was to evaluate the antifungal activity of chitosan upon some dermatophytes (*T. rubrum* and *M. canis*) and some *Aspergillus* (*A. terreus, A. niger, A. flavus* and *A. fumigatus*).

For this, the MIC of chitosans was determined and the results showed that chitosan possesses antifungal activity against *T. rubrum* and *M. canis,* presenting MIC ranging from 1.1 to 2.2 mg/mL. For *Aspergillus* species, in the range of tested concentrations it was not possible to determine the chitosan's MICs. Minimum fungicidal concentrations were also obtained for both dermatophytes, corresponding in both cases to the values obtained for MIC.

As dermatophytes are responsible for infections of keratinized surfaces, is crucial to understand if chitosan exert any effect on fungi activity. The analysis of SEM images showed that chitosan seems to have a protective effect of the hair (substract), when this was infected with dermatophytes.

The study of the effect of chitosan on enzymatic activity using protease K showed an important activity in preventing proteases action. The effect of chitosan on keratin degradation by *M. canis* and *T. rubrum*, was also studied by keratin-azure test and the results showed that the dye release is reduced when chitosan is present in culture media.

A microscopic analysis of keratin-azure surface when chitosan was present in the culture media corroborated the previous conclusion, because keratin-azure surface treated with chitosan showed no damage, despite the existence of fungal structures around them.

Based on the obtained results, it´s possible to conclude that chitosan showed relevant antifungal activity against dermatophytes, which opens good prospects to chitosan as an alternative for the usual tinea treatments.

Acknowledgments

During the realization of this work, I could count with the help of many people. Reached the end, it´s time to thank you those who make this work possible.

First, as protocol dictates, I would like to acknowledge Escola Superior de Biotcnologia da Universidade Católica Portuguesa, the institution that enabled the realization of this work.

I take this opportunity to express my gratitude to my supervisors: to prof. Manuela Pintado a great thank you for all the patience and constant invaluable guidance during the realization of this thesis; to prof. Freni Tavaria, thank you for all the advice and wise words.

To all who shared the working bench with me thank you for your support. To Manuela Santos, thank you for all the help. To Catarina Fardilha thank you for your precious help with SEM and for all that crazy moments.

To my grandmother. Granny thank for always believing in me and for being so proud of my efforts.

To Sãozinha. You've been a great friend during all my life. Thank you for supporting me in good times an bad times.

This chapter of my life is ended. Thank you all for being a part of it.

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List of Abbreviations

Basal medium	BM
Deacetylation Degree	DD
Dermatophyte test medium	DTM
High molecular weight	HMW
Low molecular weight	LMW
Medium molecular weight	MMW
Minimum fungicidal concentration	MFC
Minimum inhibitory concentration	MIC
Molecular weight	MW
Rose Bengal Chloramphenicol Agar	RBCA
Sabouraud dextrose agar	SDA
Scanning electron micoscopy	SEM

1. Introduction

1.1 Dermatophytes

Dermatophytes are a group of fungi that have the ability to invade keratinized tissues of humans and other animals and produce an infection called dermatophytosis (Weitzman and Summerbell, 1995). They are hyphomycetes that are classified among the Arthrodermataceae in their perfect form (Brasch, 2010). Organisms from Arthrodermataceae family are filamentous fungi closely related to dimorphic fungi in the Onygenales, most closely to genus *Coccidioides* (Martinez *et al.*, 2012). These fungi are classified in three anamorphic genera, *Epidermophyton*, *Microsporum* and *Trichophyton* (Wagner and Sohnle, 1995).

1.1.1. Main characteristics

Some dermatophytes have a perfect form, i.e., present sexual reproduction and produce ascomata with asci and ascospores (Weitzman and Summerbell, 1995). These fungi belong to genus *Arthroderma* (Lemsaddek, 2008).

Dermatophytes have a septate mycelium in which arthrospores are formed as a result of hyphae fragmentation (Johnson, 2003).

They are resistant to cycloheximide and they increase the pH in media with glucose and peptone (Lemsaddek, 2008) because the proteolysis of dermatophytes resuls in the liberation of excess of ammonium ion (Weitzman and Summerbell, 1995).

These fungi are also highly specialized in the degradation of keratin because they all produce an enzyme, called keratinase, that degrade this protein (Weitzman and Summerbell, 1995).

In the table 1.1 are listed the main characteristics of dermatophytes.

Table 1.1 – Characteristics of dermatophytes.

	Epidermophyton	*Microsporum*	*Trichophyton*
Macroconidia	Abundant; Large; Thin wall; Multicellular; Club-shaped; Produced in clusters; Smooth surface; Broadly clavate; One to nine septa; 20 to 60 by 4 to 13 mm	Numerous or scarce; Multispetate; Thin or thick echinulate wall; Spindle shaped; Rough surface; 6 to 160 by 6 to 25 mm.	Thin walled; Cigar-shaped; One to 12 septa; Smooth surface; Singly or in clusters; 8 to 86 by 4 to 14 mm.
Microconidia	Absent	Less abundant than macroconidia Pyriform; 2-3 µm;	More abundant than macroconidia; Globose, pyriform or clavate, or sessile or stalked; Borne singly along the sides of the hyphae or in grape-like clusters. Some species rarely produce them
Model organism	*E.flocosum*	*M. audouinii*	*T. tonsurans*
Colonies aspect	Slow-growing; powdery; brownish yellow	Powdery or velvety; White to brown pigmentation;	Powdery, velvety or waxy; Reverse pigmentation characteristic from the species and used in identification

Adapted from Simpanya, 2000, Lakshmipathy, *et al.*, 2010, Weitzman and Summerbell, 1995 and Lemsaddek, 2008.

So far, several species have been described, including 2 species in *Epidermophyton*, 20 in *Microsporum* and 26 in *Trichophyton* genus. All of them are listed in the table 1.2.

Table 1.2 – Genus and species of dermatophytes.

Epidermophyton	***Microsporum***	***Trichophyton***
E. floccosum	*M. amazonicum*	*T. ajelloi*
E. stockdaleae	*M. appendiculatum*	*T. concentricum*
	M. audouinii	*T. eboreum*
	M. boullardii	*T. equinum*
	M. canis	*T. fischeri*
	M. cookei	*T. flavescens*
	M. distortum	*T. georgiae*
	M. duboisii	*T. gloriae*
	M. equinum	*T. gourvilii*
	M. ferrugineum	*T. kanei*
	M. fulvum	*T. langeronii*
	M. gallinae	*T. megninii*
	M. gypseum	*T. melis*
	M. langeronii	*T. mentagrophytes var. erinacei*
	M. nanum	*T. mentagrophytes var. goetzii*
	M. persicolor	*T. mentagrophytes var. granulosum*
	M. praecox	*T. mentagrophytes var. interdigitale*
	M. racemosum	*T. mentagrophytes var. krajdenii*
	M. ripariae	*T. mentagrophytes var. mentagrophytes*
	M. rivalieri	*T. mentagrophytes var. nodulare*
		T. mentagrophytes var. quinckeanum
		T. raubitschekii
		T. rubrum
		T. sarkisovii
		T. schoenleinii
		T. simii
		T. soudanense
		T. terrestre
		T. tonsurans
		T. vanbreuseghemii
		T. verrucosum
		T. verrucosum var. autotrophicum
		T. violaceum
		T. yaoundei

Adapted from Lemsaddek, 2008.

1.1.2. Ecology

In the course of the evolution, dermatophytes have developed host specificity. Based on their host specificity, these fungi are classified into three ecological groups: geophyles, zoophiles and anthropophiles.

The geophilic dermatophytes are usually saprophytic and obtain nutrients from keratinous substrates. These fungi rarely cause infection in animals and humans.

Zoophiles can infect humans. They are pathogens with only one animal host and grow as saprophytes on animal materials.

Anthropophiles dermatophytes infect humans, as they are their primary host, but they also can cause infection in animals. Transmission of the infection is from man to man (Lakshmipathy and Kannabiran, 2010).

The passage of the saprophytic way of life in the soil to an almost exclusively human parasitism way was accompanied by a decrease or loss of conidial formation as well as the failure of sexual reproduction. Along with the loss of the ability to reproduce sexually, anthropophilic species tend to produce chronic infections that are difficult to resolve spontaneously (Lemsaddek, 2008).

1.2 Dermatophytosis

Infections caused by dermatophytes are called dermatomycoses, dermatophytosis, ringworm or *tinea*, and they are infections of the skin, hair and nails caused as a result of colonization of keratinazed tissues of the body. (Lakshmipathy and Kannabiran, 2010).

Dermatophytosis are cosmopolitan, contagious mycoses that infect a wide range of mammals (including man) and more rarely birds. Dermatophytes that infect animals are mostly zoophilic, but can also be geophilic and exceptionally anthropophilic (Chermette *et al.*, 2008).

Dermatophytosis can occur on people of any age. Among the *tinea* infections, *tinea corporis*, *tinea cruris*, *tinea pedis* and *onychomycosis* are the most predominant types (Lakshmipathy and Kannabiran, 2010).

There are, also, geographic patterns concerning the infection. Tinea pedis is more common in developed countries, while tinea capitis is more common in developing countries (Martinez et al., 2012).

There are different types of *tinea* and their names are given according to the site of infection (see table 1.3).

Tinea barbae refers to the infection of the bearded area, neck and mustache. So, it only occurs in adult male. It may be superficial or a severe inflammatory pustular folliculitis. If, this infection occurs in others places of the face or in children and women, it´s called ***tinea faciei***.

Tinea capitis is the infection of the scalp but hair, skin, eyelashes and eyebrows can, also, be affected. There are two types of hair infection: ectothrix, in which arthroconidia formed outside the hair shaft, and endothrix where arthroconidia formed within the hair shaft.

Tinea corporis or circinata is the ringworm of the body and usually involves the trunk, shoulders or limbs and occasionally the face. The infection appears as annular, scaly patches with sharply marginated, raised erythematous vesicular borders and may range from mild to severe.

Tinea cruris is the infection of the groin, perianal and perineal areas and, sometimes, the upper thighs and is usually seen in adult men. According to Weitzman and Summerbell (1995), the lesions are erythematous to tawny brown and covered with thin, dry scales. They are commonly bilateral and often asymmetric, exhibiting a raised, sharply marginated border that is frequently studded with small vesicles. They extend down the sides of the inner thigh.

Tinea favosa is a severe and chronic disease characterized by the presence on the scalp and glabrous skin of yellowship, cup-shaped crusts, which are composed of epithelial debris and dense masses of mycelium.

Tinea imbricata is a chronic infection of the skin. It´s characterized by concentric rings of overlapping scales scattered throughout the body (Weitzman and Summerbell, 1995; Lemsaddek, 2008) .

Tinea manuum is the infection of the palmar and interdigital areas of the hand caused by dermatophytes. Frequently this is presented as unilateral diffuse hyperkeratosis with accentuation of the flexural creases.

Tinea pedis, also called athlete's foot, is the dermatophytes' infection of the feet, especially the toes and toes webs. The most common clinical manifestation is the intertriginous form. The symptoms of this manifestation are maceration, peeling, and fissuring, mainly in the spaces between the fourth and fifth toes. Another common presentation is the chronic, squamous, hyperkeratotic type. In this manifestation fine silvery scales are formed with cover pinkish skin of the soles, heels and sides of the foot.

Tinea unguim is the invasion of the nail plate by a dermatophyte. There are two main types of nail involvement: invasive subungual (distal and proximal) and superficial white mycotic infection (leukonychia trichophytica). In the last one, the fungus infects the superficial layer of the nail (Weitzman and Summerbell, 1995, Lemsaddek, 2008).

Folicular tinea may occur when a cutaneous dermatophyte infection is exposed to occlusion or repeated superficial trauma. The lesions presents as an erythematous plaque with follicular papules or pustules. Erythema may be deeper than classic tinea coporis.

Tinea incognito is *tinea corporis* but the lesions suffer some modifications due to the application of mild to high potency corticosteroids. In most cases, there is a diffuse erythema with follicular papules and pustules due to the annular scalling and the circumscribed borders are absent (Degreef, 2008).

Table 1.3. – Types of *tinea* considering the affected areas and responsible fungi.

Tinea	**Affected body area**	**Responsible fungi**
Barbae* (barbers' itch)/ *faciei	Bearded region of face and neck/ other areas of the face or the same areas as *tinea barbae* but in women and children	*Trichophyton verrucosum* *T. mentagrophytes var. mentagrophytes* *T. mentagrophytes var. erinacei*
Capitis	Scalp	*Trichophyton sp.* *Microsporum sp.*
Corporis or circinata	Non-hairy, glaborous region of the body	*Trichophyton sp.* *Microsporum sp.* *Epidermophyton sp.*
Cruris	Groin	*Trichophyton rubrum* *Epidermophyton floccosum*
Favosa	Hair and skin	*Trichophyton schoenleinii*
Imbricata	Modified from *tinea corporis*	*Trichophyton concentricum*
Manuum	Hand	*Trichophyton rubrum*
***Pedis* (athletes' foot)**	Foot	*T. mentagrophytes* *T. mentagrophytes var. interdigital* *T. rubrum* *E. floccosum.*
Unguim	Nail	*T. rubrum* *T. mentagrophytes*
Folicular	Same as *tinea corporis*	*T. rubrum*
Incognito	Same as *tinea corporis*	*T. rubrum*

Adapted from: Weitzman and Summerbell, 1995, Lemsaddek, 2008 and Degreef, 2008

Fig.1.1 – Types of *tinea*: A - *Tinea barbae*; B- *Tinea capitis*; C- *Tinea capitis* endotrix; D- *Tinea capitis* ectotrix; E- *Tinea corporis*; F- *Tinea cruris*; G- *Tinea favosa*; H- *Tinea imbricata*; I- *Tinea manuum*; J- *Tinea pedis*; K- Subungual distal *tinea unguium*; L- Subungual proxima *tinea unguium*; M- White mycotic *tinea unguium*. Adapted from Lemsaddek (2008).

1.2.1. Infection of keratinazed areas

Dermatophytes are not part of the normal human skin flora, but they are well adapted to this infection site because they can use keratin as a source of nutrients, unlike other fungal pathogens (Wagner and Sohnle,1995).

They enter in the organism possibly through injured skin, scars and burns. The infection is caused by arthrospores or conidia. The fungus invade the uppermost, non-living, keratinazed layer of the skin – stratum corneum – and produces the exo-enzime keratinase, inducing the inflammatory reaction at the site of the infection (Lakshmipathy and Kannabiran, 2010).

The first step is the entrance of fungal elements capable of germination into the skin or at least adherence of such elements to the stratum corneum. When vital fungal elements attach long enough to the stratum corneum, they germinate, hyphae develop and spread centrifugally.

Dermatophytes release several (extracellular) enzymes during growth. These enzymes allow them to degrade and utilize keratins, other proteins, lipids and DNA as nutrient sources (Brasch, 2010) supporting them in infection process. Some of more relavant enzymes are described following.

1.2.1.1 Proteases

Proteases are one type of relevant extracellular enzymes produced by these fungi . Proteases are all enzymes that catalyse the cleavage of peptic bonds of proteins, digesting them into peptides or free amino acids. They can be divided into endoproteases and exoproteases. The first ones cleave peptide bonds within a polypeptide and the second ones cleave polypeptides only at the N- or the C-terminus of the chain (Monod, 2008).

Dermatophytes possess a genome encoding a battery of secreted proteases similar to that of *Aspergillus* species (Monod, 2008).

Proteases are released from the mycelium and they can hydrolyse many soluble proteins. Some proteases are secreted on culture medium constitutively, particularly when growth begins. However, in the exponential phase, it´s verified a decrease in proteases' production due to the lack of carbon, nitrogen and sulphur sources. Extracellular proteases of dermatophytes are neutral or alkaline proteases. Their optimum pH is in the range of 6 to 9

(Kunert, 2000).

In dermatophytes, proteolytic and keratinolytic activities aren´t lower in opportunistic species when compared to obligate parasites. However, there are differences in proteolytic activity among strains isolated from different types of lesions (Kunert, 2000).

Dermatophytes also secrete multiple serine and metallo-endoproteaes called subtilisins and fungalysins, respectively (Vermount *et al.*, 2008). These enzymes are also known as keratinases. Viani *et al.* (2001) established a direct relationship between keratinases and pathogenicity. Dermatophytes fungalysins are glycoproteins with a molecular mass of 40-80 KDa (Monod, M., 2008).

Proteases are released from the fungus mycelium on most culture media (Kunert, 2000). However, they are produced at high levels when the available carbon and nitrogen source consists in complex proteins instead of glucose or peptidic digests (Vermount *et al.*, 2008) .

Proteolytic enzymes, specially keratinases, are partly responsible for dermatohytes' ability to invade skin and disseminate through stratum corneum. Because of this, they have been the most studied enzymes (Ghahfarokhi *et al.*, 2003). One of the methods to study keratinases is to determine the keratinolytic activity of these enzymes by measure the release of soluble proteins, peptides and amino acids from keratinaceous substrates (Kunert, 2000). Other method consists in measure the clear zone around fungal colonies grown on media with keratin (Wawrzkiewicz *et al.*, 1991, Ghahfarokhi *et al.*, 2003)

1.2.1.2 Lipases and esterases

Dermatophytes can produce lipases and esterases and this production was demonstrated by plate tests, diagnostic kits and on liquid media. Lipases and esterases production is usually studied in media containing lipids but, they are not only produced in these media. They are also produced in media with keratin and even in Sabouraund glucose-peptone broth.

There are usually great differences in lipolytic activity between species and strains of dermatophytes but it is usually moderate to weak. For instance, *T. rubrum* has a weak lipolytic activity (Kunert, 2000).

1.2.1.3 Other enzymes

Dermatophytes produce an extracellular phosphatase with wide specificity and optimum pH around 8.7. The enzyme is secreted constitutively because it was found in media containing inorganic phosphate (Kunert, 2000).

Amylase is also produced by these fungi and some authors have described the starch utilization by and amylase production. Dermatophytes can't hydrolyse pectins, native cellulose and its derivates and products. However, they are able to hydrolyse simple sugars (Kunert, 2000).

Enzymes are not the only tools that allow dermatophytes to degrade keratinized subtracts. By excreting large quantities of sulphite – sulphitolysis - dermatophytes can cleave dissulphide bridges. This way, reduced proteins become accessible for further digestion by proteases (Monod, 2008).

During the the skin invasion by dermatophytes they do not have only the ability to produce enzymes or the excretion of sulphite. These fungi are able to invade the stratum corneum because there are a number of conditions in the skin that favour their growth. These favourable conditions are:

1) the stratum corneum is an avascular tissue composed of highly specialized but dead cells. So, it´s distant from the body mains defensive mechanisms.

2) the stratum corneum is well hydrated so, the skin temperature is cooler than body temperature, pH ranges from 5.5 to 6.7, and skin is exposed to the aerobic conditions of the atmosphere.

3) stratum corneum is favourable to dermatophytes' growth because it's composed of proteins, amino acids, lipids, carbohydrates and various trace elements, including iron.

4) in some areas of the stratum corneum there are certain anatomical considerations which may enhance establishment of growth of dermatophytes: hair on scalp may act as a trapping device for an airborne dermatophyte infection; the hyponychial horny

layer is covered by the distal portion of the nail plate and a groove is thus constructed which may also act as a trapping device for dermatophyte infective particles; the interdigital spaces of the toes and the crural areas in males are naturally occluded, which may explain the fact that *tinea* pedis in most instances starts in the toes webs and *tinea* cruris is almost exclusively a male disease (Richardson and Edward, 2000).

The mechanism by which keratinophilic fungi degrade keratin is a result of mechanical action of the fungus and the enzymatic activity of intra cellular keratinase (Ali-Shtayeh and Jamous, 2000).

Dermatopytes induce the normal signs of inflammatory reactions such as ruber (redness), induration (swelling), heat and alopecia (loss of hair). Inflammation causes the pathogen to move away from the infection's site and fix in a new site. This movement of the fungus produces the classical ringed lesion (Lakshmipathy and Kannabiran, 2010).

1.2.2. Impact and epidemiology of dermatophytosis

Animal dermatophytosis is of great concern in public health as the majority of dermatophytes isolated from animals are zoonotic (Chermette *et al.*, 2008).

Dermatophytoses appear commonly in pet animals, in livestock and sometimes in wildlife. Because it is a contagious disease, there is a high occurrence of *tinea* in herds and animal collectivities. Although, it´s not a serious illness, it has serious economic consequences due to the long duration of the disease, the easy contamination and spread of infection among animals and the difficulties and costs of control measures. Because *tinea* is a contagious disease and due to unaesthetic aspect of the lesions, it is an obstacle the attendance at pet exhibitions and sport activities for horses, to commercial transactions and animal commerce.

Furthermore, *tinea* also leads to losses in hide and skin industry as scars resultants from *tinea*' lesions reapper on leather at tawing and tannery (Chermette *et al.*, 2008).

Considering humans dermatophytosis, these infections are the most common cause of fungal infections worldwide, affecting millions of people annually. On United States, they have an economic impact on health care system that exceed $400 million a year (White *et al.*, 2008)

All genera of dermatophytes have a worldwide distribution, excluding Antarctida (Lakshmipathy and Kannabiran, 2010; Martinez *et al.*, 2012). The distribution of dermatophytes presents a great variance around the world. In Europe, particularly, in Mediterranean countries and in Slovenia, the incidence of *M. canis* infections has increased during the recent past years. *T. rubrum* is the main cause of *tinea* pedis, nail infections, tinea cruris and tinea corporis in the world. These fungi are replacing other dermatophytes. This fact is probably related to the many possibilities of infection by *T. rubrum* due to its huge distribution in the population of many places (Seebacher *et al.*, 2008).

In developed countries, the incidence of *tinea* capitis is decreasing but, *tinea* pedis and onychomycosis are becoming an epidemiologic and economic problem (Seebacher *et al.*, 2008). The three main factors for the current distribution of dermatophytes in Europe, according to Svejgaard (1998), are: 1) the existence of a poor living standard in several Eastern and Southern European countries is responsible for the increase in zoophilic infections; 2) the increase of the spread of *T. rubrum* seems to be related to urban areas with dense populations and social activities, like travelling and sports; 3) the increase of people´s migrations leads to the reintroduction of other antropophilic species.

1.2.3. Treatment of dermatophytosis

The treatment of dermatophytosis begins with topical antifungals. These antifungals should penetrate the skin and remain there in order to suppress the fungus (del Palacio *et al.*, 2000).

The antifungal agents used in dermatophytosis treatments can´t be irritant and must be well tolerated (del Palacio *et al.*, 2000). In addition, these drugs should selectively kill the fungal cells without affecting the other cells (Subha and Gnanamani, 2009).

The primary treatment indicated for *tinea corporis*, *tinea cruris*, *tinea pedis* and *tinea manuum* consists in topical medications. There are many topical agents available in cream, gel, lotion and shampoo formulas. The agents from azole antifungal family (e.g. clotrimazole, miconazole, econazole, oxiconazole) are the most used. Agents from the allylamine family, as terbinefine and naftifine, are also used (Gupta and Cooper, 2008).

Oral therapies are the primary treatments in case of *tinea unguim* (onychomycosis) and *tinea capitis*. However, there is some evidence suggesting that in less severe cases of *onychomycosis*, a topical treatment can be effective.

The most common topical preparations used for *tinea pedis* and *tinea manuum* are formulations of terbinefine, butenafine, miconazole, econazole, ketoconazole, clotrimazole, oxiconazole and ciclopirox. If the infection is chronic, oral antifungals may be used.

Terbinefine, butenafine, econazole, miconazole, ketoconazole, cloritromazole and ciclopirox are some of the topical therapies for treatment of *tinea corporis* and *tinea cruris*. However, topical therapies can eradicate only small infected areas. So, to treat larger areas or when the infection is chronic or recurrent, oral therapy may be needed. To treat *tinea capitis*, oral therapy is required, because it can penetrate the hair shaft and topical therapies cannot.

However, topical antifungals can be used to prevent reinfections or treat asymptomatic carriers. *Tinea unguium* is difficult to cure. Finger nails are usualy easier eliminated than toe nails, because they grow faster and the doses of antifungal used are smaller. In subjects with *tinea unguim*, nail debridement may be a complement to antifungal therapy. Sometimes is necessary to remove the nail plate surgically to eliminate most of the living fungi (Gupta and Cooper, 2008).

In some cases of dermatophytosis, bacterial infections may be present. Because of this, antimicrobial formulation combining both antibacterial and antifungal action may be useful (Gupta and Cooper, 2008).

The control of dermatophytic infections requires prolonged therapy, and the existing drugs, on long run seem to exhibit side effects, which requires new drug formulations for long term use that are biocompatible (Subha and Gnanamani, 2009) and with less secondary effects.

1.3. *Aspergillus* and aspergillosis

Aspergillus species are largely distributed in the environment and are often associated with rotting vegetation. However, they are human opportunistic pathogens that can cause primary invasive lung infections and disseminate to other organs (Cannon *et al*., 2009).

The main cause of aspergillosis is *Aspergillus fumigatus* (85%), *Aspergillus flavus* (5 to 10%) and *Aspergillus terreus* (2 to 3%). *Aspergillus niger* (2 to 3%), *Aspergillus nidulans* and *Aspergillus ustus* are rarely isolated (Cannon *et al.,* 2009).

Invasive aspergillosis is associated with high mortality in immunocompromised patients, like those with leukemia and marrow transplants (Tarrand *et al.*, 2005). Inhalation of fungal conidia is thought to be the primary cause of acquiring aspergillosis (Fridkin and Jarvis, 1996).

Cutaneous infections caused by *Aspergillus* spp. have been associated with the use of arm boards in patients with intravascular catheters and with bandages or dressings used for these catheters (Fridkin and Jarvis, 1996).

1.4 Chitosan

Chitosan is a cationic polysaccharide composed of β-1,4 linked D-glucosamine and N-acetyl-D- glucosamine residues (Meng *et al.,* 2012). It only occurs naturally in some fungi (Mucoraceae). On the other hand, chitin is the most abundant polymer in nature after cellulose (Aranaz *et al.,* 2009). It´s found predominantly in the shells of crustaceans, the cuticles of insects and the cell walls of fungi (Kumirska *et al.,* 2011). Figure 1.2 shows the structure of chitin and chitosan.

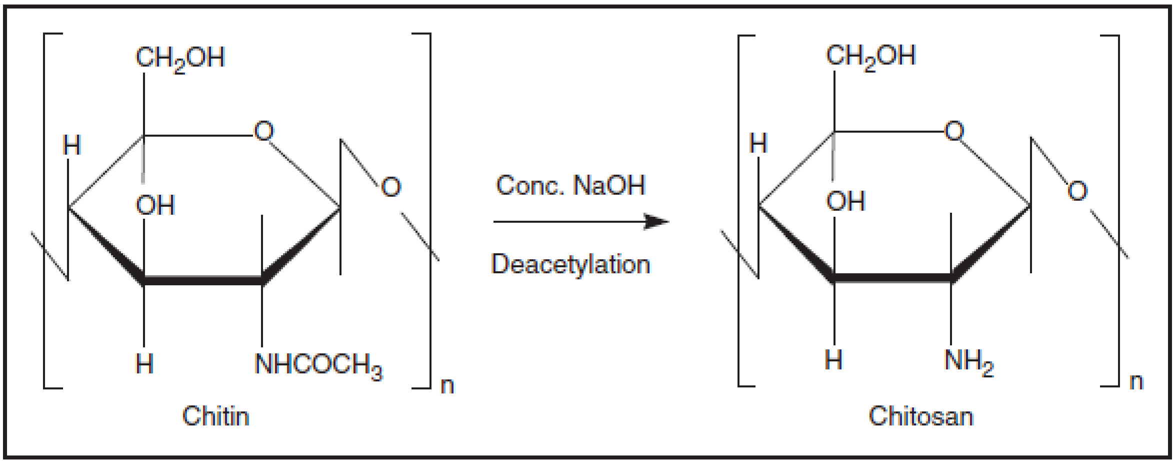


Fig.1.2 – Structrure of chitin and chitosan. Adapted from Dutta *et al.* (2011).

Chitosan can be prepared by cleavage of N-acetyl groups in chitin N-acetyl-2 amino-2-deoxy-D-glucose residues, requiring several steps.

First, chitin is extracted by acid treatment to dissolve calcium carbonate followed by alkaline extraction to dissolve proteins. Then, a depigmentation step is carried out for removing the astaxantine, allowing a colourless product. After that, a severe alkaline hydrolysis is carried out in order to hydrolyse the acetamine groups of chitin and obtain the chitosan (Aranaz et al., 2009).

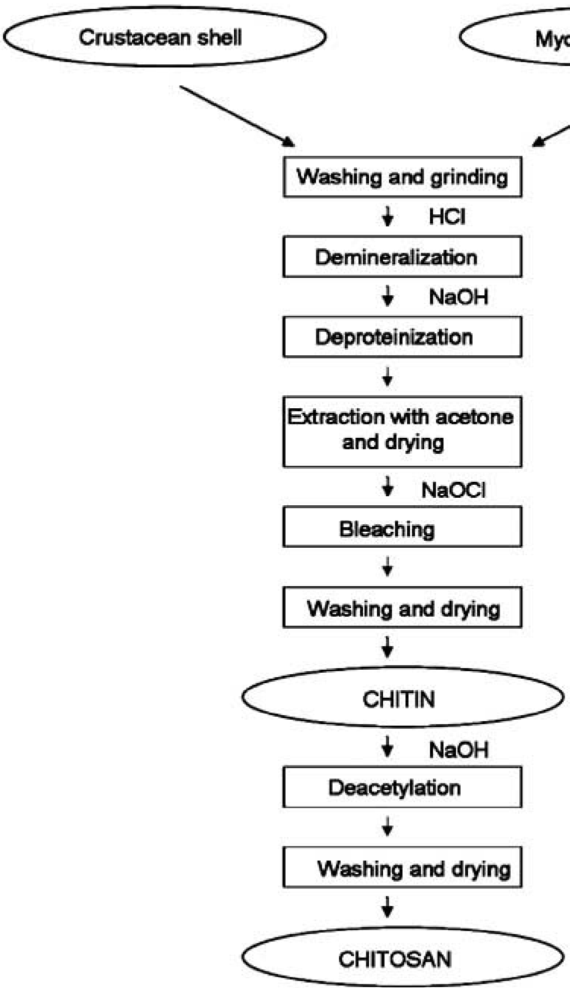


Fig.1.3 – Mode of preparation of chitin and chitosan. Adapted from Aranaz *et al.* (2009)

1.4.1. Properties of chitosan

Chitosan has proved to be non- toxic, biodegradable, biocompatible, and to possess several biological properties, including: antimicrobial, anticholesterolemic, antioxidant, anti-inflammatory, antitumoral, analgesic and haemostatic activities, mucoadhesion capacity, angiogenesis stimulation, macrophage activation, granulation and scar formation, and adsorption enhancer (permeation enhancer) (Aranaz *et al.,* 2009).

Chitosan is a **non-toxic** polymer. A proof of that is its utilization in dietary applications in Japan, Italy and Finland and the approval by the FDA for use in wounds dressings (Dash *et al.*, 2011).

Chitosan is **biodegradable** because it is not present in mammals, but it can be degraded by several enzymes (Aranaz *et al.,* 2009). The degradation may occur by non-specific enzymes such as lysozymes, chitinases, cellulases or hemicellulases, proteases, lipases and β-1,3 -1,4-glucanases.This polymer is also hydrolysed by chitosanases; these enzymes attack chitosan but not chitin, catalysing the endohydrolysis of β- (1→4)- glycosidic linkages between D-glucosamine residues in partly acetylated chitosan (Raafat and Sahl, 2009).

It is also **biocompatible**. According to Knapczk *et al.* (1984) chitosan has a LD_{50} of around 16 g/kg, which is very similar to salt and glucose values for mice. Chitosan's biocompatibility depends on deacetylation degree. Chitosan samples with a deacetylation degree higher than 35% showed low toxicity, while chitosans with a deacetylation degree under than 35% present dose dependant toxicity (Aranaz *et al.,* 2009).

This polymer has proved also to be **haemostatic**. This property seems to be related to chitosan's positive charge since red blood cells´s membranes are negatively charged (Aranaz *et al.,* 2009).

Another property of chitosan is **analgesic effect**. The main cause for the analgesic effect of chitosan is the reduction on the pH due to the free primary amino groups of chitosan that can protonate in the presence of proton ions (Aranaz *et al.,* 2009).

Additional, it has been claimed that chitosan can inhibit the growth of tumour cells mainly due to an immune stimulation effect. Studies carried out using mice that had ingested low-Mw chitosan revealed significant antimetastatic effects of chitosan against Lewis lung carcinoma. This demonstrate the **antitumor activity** of chitosan (Aranaz *et al.,* 2009).

Mucus is composed of mucin, which is rich in negative charges. In stomach, chitosan has positive charge due to the acidic environment and can interact with mucin. This interaction depends on the amount of sialic acid present in the mucin and on the molecular weight and deacetylation degree of chitosan. This binding affinity of chitosan explains its **mucoadhesion** (Aranaz et al., 2009).

This polysaccharide has, also, the ability of **activate macrophages.** This property is related to antitumor activity because it has suggested that the activation of macrophages mediate the antitumor effects *in vivo*. Chitosan possess **angiogenic inducing** properties, too.

Nevertheless, this is a harmful effect of chitosan because it may promote tumour growth and invasion (Aranaz *et al.,* 2009).

Chitosan acts as a **permeation enhancer** because it can open the epithelial tight junctions. The mechanism that explains this behaviour is based on the interaction of positively charged chitosan and the cell membrane, which results in a reorganization of the tight junction-associated proteins.

Chitosan is an **anticholesterolemic** substance. There are several mechanisms proposed to explain cholesterol reduction by chitosan. One of those is reduction of absorption of fat and cholesterol by the entrapment caused by viscous polysaccharide solutions; other is the presence of the amino group in chitosan's structure which determines the electrostatic force between chitosan and anion substances, such as fatty acids and bile acids (Aranaz *et al.,* 2009).

Chitosan shows **antimicrobial activity** against different groups of microorganisms, such as bacteria, fungi and yeasts. There are many mechanisms that can explain this chitosan's property: 1) the interaction of chitosan with anionic groups on cell surface, due to its polycationic nature, causes the formation of an impermeable layer around the cell, which prevents the transport of essential solutes; 2) the inhibition of the RNA and protein synthesis by permeation into the cell nucleus; 3) chelating agent that render metals, trace elements or essential nutrients, which become unavailable for the organism to grow at the normal rate; 4) interaction with flocculate proteins.

A last, another property of chitosan is its **antioxidant activity**. Chitosan presents a scavenging capacity against different radial species (Aranaz *et al.,* 2009).

Chitosan's properties depend on several characteristics, including degree of N-acetylation (DA), molecular weight (Mw), pH and concentration.

In table 1.4 are represented the relationships between chitosan's properties and chitosan's characteristics.

Table 1.4 – Influence of degree of N-acetylation, molecular weight and pH on biological properties of chitosan. ↑ means directly proportional to the property and ↓ means inversely proportional to the property.

Biological property	**Degree of N-acetylation**	**Molecular weight**	**pH**	**References**
non- toxicity				
biodegradability	↓			Aranaz *et al.* 2009
biocompatibility	↓			Aranaz *et al.* 2009
citocompatibilty	↑			Aranaz *et al.* 2009
antimicrobial activity	↓	↓ or ↑		Kumirska *et al.,* 2011
anticholesterolemic activity		↓		Kumirska *et al.,* 2011
antioxidant activity	↓	↓		Kumirska *et al.,* 2011; Aranaz *et al.* 2009
anti-inflamatory action	↓	↓		Kumirska *et al.,* 2011; Aranaz *et al.*, 2009
antitumoral activity		↓		Kumirska *et al.*, 2011
analgesic action			↓	Aranaz *et al.* 2009
haemostatic action	↓			Kumirska *et al.*, 2011; Aranaz *et al.* 2009
mucoadhesion	↓	↑		Kumirska *et al.*, 201; Aranaz *et al.,* 2009
macrophage activation	↑			Aranaz *et al.*, 2009
granulation and scar formation	↑	↓		Aranaz *et al.,* 2009
adsorption enhancer	↓	↑		Kumirska *et al.,* 2011; Aranaz *et al.* 2009

1.4.2. Chitosan's aplications

Due to its properties, chitosan has many applications. Some of those applications are listed in the table 1.5.

Table 1.5 – Chitosan's applications. Adapted from Aranaz *et al*., 2010

Biomedicine/pharmacy	Wound dressing
	Drug delivery systems
	Gene delivery
	Tissue engineering
	Orthopaedic, periodontal applications
	Excipient
	Radiopharmaceuticals
Cosmetics	Maintain skin moisture
	Protect epidermis
	Acne treatment
	Reduce static electricity in the hair
	Tone skin
Technology	Biocatalyst (enzyme/ cell immobilization)
	Water engineering
	Molecular imprinting
	Metal reduction, nanoparticle stabilization,
	Photography
	Textiles
	Nanomaterials
	Biosensors
	Heterogeneous catalyst
Food industry	Dietary ingredient
	Food preservation (antioxidant, antimicrobial)
	Emulsify agent
Agriculture	Gene elicitor
	Antibacterial
	Seed coatings
	Increases blooms and extends the life of cut flowers

1.5 Objectives

The general objective of this work was to evaluate the antifungal activity of selected chitosans upon some dermatophytes and some selected species of *Aspergillus* sp.

The specific objectives include:

(i) Determine the Minimum Inhibitory Concentration (MIC) of low, medium and high molecular weight chitosans upon some dermatophytes and some selected species of *Aspergillus* sp;

(ii) Evaluate the antifungal action of chitosan against dermatophytes in a keratin matrix;

(iii) Understand the chitosan's action over some virulence factors of dermatophytes (keratinases, mainly);

(iv) Analyse structural characteristics (via optical and scanning electron microscopy) of the keratinased matrix infected with dermatophytes, treated and not treated with chitosan.

2. Materials and methods

2.1 Test organisms and media

Aspergillus terreus (A. terreus), Aspergillus flavus (A.flavus) and *Aspergillus niger (A. niger)* were spread on Rose Bengal Chloramphenicol Agar (RBCA, Lab M, Lancashire, UK) and incubated for 7 days at 30 ºC while *Aspergillus fumigatus (A. fumigatus)* was spread on Sabouraud dextrose agar (SDA, Difco, NJ, USA) for 7 days at 30 ºC and *Microsporum canis (M. canis)* and *Tricophyton rubrum (T.rubrum)* were spread on Dermatophyte test medium [DTM: SDA with 0.055% L(+) tartaric acid (Sigma, St. Louis, MO, USA), cycloheximide (0.4 g/L, Sigma), chloramphenicol (0.05 g/L, Sigma) and 0.2 g/L phenol red] for 14 days at 30ºC.

2.2. Chitosan types and preparation

High, medium and low molecular weight chitosans (MW) were obtained from Sigma-Aldrich. High MW chitosan presented a DD > 75% and a MW of 624 kDa; medium MW chitosan presented a DD between 75 and 85% and a MW of 591 kDa; low MW chitosan presented a DD between 75 and 85% and a MW of 107 kDa.

Chitosan powders were dissolved in a 0.5% (v/v) acetic acid solution and left stirring overnight until it had a gel-like consistency at room temperature. Then, the pH was adjusted to 5.6 with 5 M NaOH solution and autoclaved at 120 ºC for 15 min.

2.3 Microdilution method

High, medium and low MW chitosans were tested against *A. terreus*, *A. fumigatus*, *A. flavus*, *A. niger*, *M. canis* and *T. rubrum*.

Inocula were prepared by covering the cultures of *A. terreus*, *A. fumigatus*, *A. flavus*, *A. niger*, *M. canis* and *T. rubrum*. with 0.1% tween 20 (Sigma) solution and the colonies were gently scraped from the surface with the tip of a glass Pasteur pipette. Spores in the inocula were counted in a Neubauer chamber and the concentration was adjusted to 10^6 spores/mL.

After that, the chitosan solutions were diluted in culture medium. Five different dilutions of each chitosan were made and all of them were added in triplicate to a 96-well microplate. The chitosan concentrations tested are indicated in the table 2.1.

Table 2.1 – Concentrations of chitosan used in the microdilution method.

Concentrations of chitosan tested (mg/mL)						
	A. terreus	***A. niger***	***A. flavus***	***A. fumigatus***	***T. rubrum***	***M. canis***
High molecular weight	7.5 – 2.5	7.5 – 2.5	7.5 – 2.5	7.5 – 2.5	5.0 – 0.31	5.0 – 0.31
Medium molecular weight	13.1 – 4.2	13.1 – 4.2	13.1 – 4.2	13.1 – 4.2	6.25 – 0.39	6.25 – 0.39
Low molecular weight	13.1 – 4.2	13.1 – 4.2	13.1 – 4.2	13.1 – 4.2	8.75 – 0.55	2.2 – 0.1

The preparation of chitosan dilutions involved the use of different chitosan and culture medium volumes. Table 2.3.2 describes an example of how chitosan's dilutions were made:

Table 2.2 – Example of chitosan dilutions preparation from a stock solution for *M. canis*.

Stock solution (mg/mL)	Chitosan volume (mL)	Desired concentration (mg/mL)	Eppendorf volume (mL)	Medium volume (mL)
20	0.38	5.0	1.5	1.13
20	0.19	2.5	1.5	1.31
20	0.09	1.3	1.5	1.41
20	0.05	0.6	1.5	1.45
20	0.02	0.3	1.5	1.48

The culture medium used was potato dextrose broth for *A. terreus, A. flavus, A. niger* and Sabouraud dextrose broth for *A. fumigatus* and Dermatophyte Test Medium (DTM) for dermatophytes, as previously mentioned. A column of the microplate was used as a positive control (culture medium inoculated with fungus), another was used as a negative control (culture medium only) to verify if sterile conditions were maintained, two lines were used with chitosan controls and four wells were used as relative controls (acetic acid with medium and inoculum). These controls were carried out in order to understand if acetic acid was not affecting fungal growth. The plates were incubated at 25 ºC for 4 days for *A.terreus, A. flavus, A.niger* and *A. fumigatus* and 14 days for dermatophytes under the same incubation conditions.

The MIC was defined as the lowest concentration where no growth was observed.

In order to determine the Minimum Fungicidal Concentration (MFC), 100 µL of sample from each well where the fungus didn't grow were inoculated in Petri plates with DTM. The Petri plates were incubated at 30 ºC during 14 days. The minimum fungicidal concentration was defined as the lowest concentration in which no visible growth was observed.

2.4 Hair infection - Scanning Electron Microscopy

2.4.1 Preparation of inoculum and infection of human hair

T. rubrum and *M. canis* were cultured on DTM agar for 14 days. The inocula were prepared by covering the cultures with distilled water and the colonies gently scraped with the tip of a glass Pasteur pipette. The number of spores in the inocula was counted with a Neubauer chamber and the concentration was adjusted to 10^6 spores/mL.

Human hair was obtained in a hairdresser. The hair was cut into 2 cm long pieces, washed and sterilized with a chloroform-ethanol 1:1 solution at room temperature for 2 h as suggested in Kanbe and Tanaka (1982). A buffer solution (pH 6.5) was prepared by dissolving 0.04g of potassium phosphate (KH_2PO_4 - Sigma) in 100 mL distilled water and sterilized.

Five treatments were prepared in sterilized tubes: negative and positive controls and hair treated with high, medium and low MW chitosans. The negative control had 50 mg of hair and 20 mL of KH_2PO_4 buffer. High MW chitosan treatment was composed of 50 mg of hair, buffer and 0.06% (v/v) high MW chitosan; medium MW chitosan treatment was composed of 50 mg of hair, buffer and 0.08% (v/v) high MW chitosan; and low MW chitosan treatment was composed of 50 mg of hair, buffer and 0.11% (v/v) low MW chitosan for *T. rubrum* and 0.06% (v/v) low MW chitosan for *M. canis.* The differences in the low MW chitosan concentrations were due to the fact that MIC values for this chitosan are different for *T. rubrum* and *M. canis*. Then, 200 µL of the spore suspension was added to each tube except for the negative control tubes.

Two replicates for each fungus, treatment and week were prepared and incubated in a shaker incubator at 80 rpm (27ºC). Hair infected with each fungus was collected after 1, 2 and 3 weeks of incubation and used in a scanning electron microscopy test. The buffer present in each tube was used in an enzymatic activity test. The tubes (with the buffer) were frozen in a – 80 ºC freezer until used.

2.4.2 Scanning electron microscopy

Hair samples infected with *T. rubrum* and *M. canis* were used for this test. Samples were obtained as explained in the section above.

The specimens were fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.2-7.3) over night. Then, the fixed sections were rinsed three times in cacodylate buffer solution (pH 7.3) for 10 min each and dried in a graded ethanol series, e.g. 10, 30, 50, 70, 80, 90 and 100%, 15 min per change. The samples were mounted onto cover slips and coated with gold. The specimens were observed in a scanning electron microscope (Jeol 5600LV, Tokyo, Japan) operated at 20kV.

2.5. Effect of chitosan action on virulence factors

2.5.1 Keratinase enzimatic activity test

Keratin azure (Sigma) was used as substrate. In this test, 5 mg of keratin azure were suspended in 1 mL of 50 mmol/L Tris-HCl (Merck) buffer (pH 8.0). Then, 1 mL of enzyme solution in buffer was added.

Control tubes with the 3 chitosans used in this study were also prepared to determine the absorbance of the chitosan itself. These tubes contained chitosan dissolved in KH_2PO_4 buffer in the same concentrations used in the tubes with fungi and 5 mg of keratin azure dissolved in 1 mL of 50 mmol/L Tris-HCl buffer (pH 8.0). The enzymatic reaction was carried out at 27 ºC with constant agitation of 200 rpm/min for 1 h. After incubation, the reaction was stopped by putting the test tubes in an ice bath. Then, reaction mixtures were filtered to remove the substrate and the chitosan. The filtrate was spectrophotometrically measured for release of the azo dye at 595 nm.

One unit (U) of keratinase activity was defined as the amount of enzyme causing a 0.01 increase in absorbance between the sample and the control at 595nm after 1 h under the conditions described by Matikevičienė *et al.* (2009). The result was taken as an average of two replicates.

Furthermore, in order to infer about the reason for the low keratinase activities in the tubes with chitosan a test with proteinase K was carried out in the same way.

First, a proteinase K solution containing 100 units/mL of enzyme was made in 50 mmol/L Tris-HCl buffer (pH 8.0). Then, 5 mg of keratin azure were suspended in 1 mL of the same buffer and 1 mL of buffer or chitosan was added. The reactions were carried out at 27 ºC with constant agitation of 200 rpm/min for 1 h. After incubation, the reaction was stopped by putting the test tubes in an ice bath. Then, reaction mixtures were filtered to remove the substrate and the chitosan. The filtrate was spectrophotometrically measured for release of the azo dye at 595 nm.

2.5.2. Keratin degradation test

A basal medium (BM) was prepared as described in Scott *et al.* (2004). The medium was autoclaved and after that low, medium and high MW chitosans were added to the medium at 0.11%, 0.08%, 0.06%, respectively for *T. rubrum* and 0.06%, 0.08% and 0.06% for *M. canis*. Then approximately 15 mL were dispensed into 25 mL sterile tubes under sterilized conditions. The tubes were cooled in an upright position.

Finely chopped keratin azure was suspended to a concentration of 4 mg/ml in BM and autoclaved. Finally, 1 ml of this overlay medium was dispensed aseptically into each tube.

The tubes were inoculated with a concentration of 10^6 spores/mL except for the negative control tubes. The spores were obtained by covering the cultures with 0.1% (v/v) tween 20 solution and scraped with a tip of a glass Pasteur pipette. Then the inocula were adjusted for the desired concentration with culture medium.

The tubes were incubated at 25 ºC and observations were made after 1, 2 and 4 weeks of incubation.

Petri plates with DTM and the same chitosan concentrations were made. Each Petri plate was filled with 25 mL of medium and 3 pieces of keratin azure were distributed into the plate. Then, 100 µL of fungal suspension was inoculated in each of the 3 keratin azure pieces.

After 4 weeks of incubation, a small piece of keratin azure with fungus of each Petri dish was placed in a glass slide and stained with lactophenol cotton blue for a better observation of the fungal structures. The samples were observed in optical microscope at a magnification of 400x.

The samples were also observed with a scanning electron microscope and sample treatment was carried out as explained above (section 2.4.2).

3. Results and discussion

3.1. Determination of chitosan antifungal activity

Dermatophyte infections are an important public health problem. Currently, there are several drugs that can be used to treat them. However, the frequent and prolonged use of these fungicide compounds are responsible for strain resistance representing a potential risk for the environment and human health (Sajomsang *et al.*, 2012).

Chitosan, as a natural compound, does not present until now such problems. Additional, antifungal action has been ascribed to this compound (Sajomsang, *et al.*, 2012). However, only a few studies have been performed on fungi, and scarce studies are found for dermatophytes (Goy *et al.*, 2009; Balicka-Ramisz *et al.*, 2005). So, it is important to find more evidences of the effectiveness of this compound on fungi, and in particular dermatophytes.

3.1.1. Determination of Minimum Inhibitory Concentration (MIC)

The MIC of high MW, medium MW and low MW chitosans upon *T. rubrum*, *M. canis* and some selected species of *Aspergillus* sp. were determined.

The method used to obtain the MIC values for the dermatophytes studied was based in two characteristics of these fungi: 1) their ability of increasing the pH in media with glucose and peptone and 2) their resistance to cycloheximide.

The medium used for this test was dermatophyte test medium (DTM). This medium contains glucose, peptone, cycloheximide and phenol red as part of its composition. Furthermore, it has originally a yellow/orange colour that changed to pink/red when there was growth. This change in colour is due to the degradation of glucose and peptone that does not occurs when another microorganism grows.

Assuming the nutritious and differential media used, in the presence of chitosan at inhibitory concentrations, dermatophytes will not grow and consequently no colour change will occur.

So, as can be seen in figure 3.1, when compared with the positive control (last column from left to right), the wells where the medium remained yellow represent the concentrations in which the fungal growth was inhibited by chitosan. The MIC value corresponds to the last wells (from left to right) in which medium remained yellow.

Since chitosan solutions are produced in neutralized acetic acid, acetic acid dissolved in culture medium was used as control. Analysing the results, although chitosan was dissolved in a 0,5% (v/v) acetic acid solution, the inhibition of fungal growth was only due to chitosan, since no inhibition was observed in acetic acid solution used as control.

Fig 3.1. Photograph of microplate used to determine MICs. The tested fungus was *T. rubrum*. MICs can be determined as they are the last wells (lower concentration) in which the medium remained yellow.

The MIC values for the tree types of chitosan are given in table 3.1.

Table 3.1 – Minimum inhibitory concentration of high MW, medium MW and low MW chitosans determined for *T. rubrum*, *M. canis* and some *Aspergillus* species

Minimum inhibitory concentration (mg/mL)			
	High MW chitosan	**Medium MW chitosan**	**Low MW chitosan**
T. rubrum	1.3	1.6	2.2
M. canis	1.3	1.6	1.1
A. fumigatus	> 7.5	> 13	> 13
A. terreus	> 7.5	> 13	>13
A. flavus	------	> 13	> 13
A. niger	-------	> 13	> 13

Note: ------- means not determined

As can be seen, MIC values are the same for high MW and medium MW chitosans for both *T. rubrum* and *M. canis*. However, for the low MW chitosan *M. canis* showed higher susceptibility. So, the most efficient chitosan for *M. canis* was the low MW chitosan (MIC value of 1.1 mg/mL) whereas high MW chitosan was the chitosan with the best result for *T. rubrum* (MIC value of 1.3 mg/mL).

It is believed that chitosan's cationic nature and high MW helps in its antifungal action, since it interferes with negatively charged residues of macromolecules on the fungal cell surface, thus causing changes in cell membrane permeability (Di Piero and Garda, 2008; Coqueiro and Di Piero, 2011). It can, also, prevent DNA transcription to RNA (Li, *et al.*, 2008).

Some authors (Li, *et al.*, 2008) mentioned that antifungal activity of chitosan is MW dependent and that the smaller the molecular weight is, the stronger will be the antifungal activity. In this work, this relation was not verified because, for *T. rubrum*, high MW chitosan has the smaller MIC whereas for *M. canis*, the lower MIC was observed for low MW chitosan. These results can be explained by the fact that antifungal activity is dependent on the fungi´s type; a fungus can interfere with antifungal activity of a drug due to its adaptation and defence mechanisms to stress, which can affect the structural integrity of the cell wall or induce the synthesis of defence coumpounds (Sajomsang *et al.*, 2012).

Other works refer that inhibitory action of chitosan is directly proportional to the concentration, because at higher concentrations of chitosan, fungi will produce higher concentrations of chitinase and this leads to the degradation of chitin and chitosan of fungal cell walls (Li, *et al.*, 2008).

This fact is possible ascertained in this study because analysis of the microplates shows that at higher concentrations of chitosan (fig. 3.1), the culture medium remained yellow (absence of fungal growth) and at the lowest concentrations, the colour of the culture medium changed from yellow to orange, pink and then red.

Additionally, to the direct inhibitory effect of chitosan on fungi growth, chitosan also possess the ability in preventing spores germination. In fact, chitosan can interfere with the uptake of minerals, particularly Ca^{2+}, and nutrients, thereby delaying the spores germination (Plascencia-Jatomea *et al.*, 2003).

In this work, the antifungal activity of chitosan against *T. rubrum* and *M. canis* was proven. Some authors also studied chitosan antifungal activity on dermatophytes. Balicka-Ramisz *et al.* (2005) obtained a MIC value of 1.1 mg/mL for *M. canis*. This is the same MIC value obtained in this thesis for low MW chitosan for the mentioned fungus. Goy *et al.* (2009) also determined the MIC values for *M. canis* and the result was 1100 ppm (i.e. 1.1 mg/mL). This is the same MIC value described by Ramisz *et al.* (2005) and the same value presented in this work for low MW chitosan. Both authors also determined the MIC of chitosan for another dermatophyte fungus, *T. mentagrophytes,* and reached the value of 2.2 mg/mL. Although MIC values for this fungi were not determined in this thesis, we cannot avoid remarking that the MIC value obtained by the two authors for this fungus is the same as the one determined in this work for *T. rubrum* when it was treaded with low MW chitosan.

But, will chitosan be more or less efficient in eradicating these fungi than others natural antifungal compounds? In order to answer this question, a comparison of chitosan's MICs obtained in this work with MICs of other natural antifungal substances was done. Those antifungal activities were obtained from other studies and listed in the following tables:

Table 3.2 – Minimum inhibitory concentration of some natural antifungals (plant extracts) for *T. rubrum*.

T. rubrum		
Antifungal drug	**MIC (mg/mL)**	**Reference**
Onion (Allium cepa)	2	Shams-Ghahfarokhi, *et al.*, 2006
Chili (*Capsicum annuum*)	9.3	Vaijayanthimala *et al.*, 2004
Coffee (*Coffea arabica*)	9.3	Vaijayanthimala *et al.*, 2004
Cumin (*Cuminum cyminum*)	9.3	Vaijayanthimala *et al.*, 2004
Turmeric (*Curcuma longa*)	4.6	Vaijayanthimala *et al.*, 2004
Curry leaf (*Murraya koenigii*)	18.7	Vaijayanthimala *et al.*, 2004
Ficus exasperata	44.64	Mbakwem-Aniebo *et al.*, 2012
Ocimum gratissimum	63.06	Mbakwem-Aniebo *et al.*, 2012
Polygonum aviculareae	100	Maoz and Neeman, 1998
Polygonum equistiforme	100	Maoz and Neeman, 1998
Cynodon dactylon	50	Maoz and Neeman, 1998
Inula viscosa	6.3	Maoz and Neeman, 1998
Prosopsis farcta	100	Maoz and Neeman, 1998
Amni visnaga	25	Maoz and Neeman, 1998
Salvia fruticosa	50	Maoz and Neeman, 1998
Celltis australis	100	Maoz and Neeman, 1998
Tamarix aphylla	100	Maoz and Neeman, 1998
Laurus nobilis	50	Maoz and Neeman, 1998

Table 3.3 – Minimum inhibitory concentration of some natural antifungals (plant extracts) for *M. canis*.

M. canis		
Antifungal drug	**MIC (mg/mL)**	**Reference**
Onion (*Allium cepa*)	2	Shams-Ghahfarokhi, *et al.*, 2006
Anise (*Pimpinella anisum*)	8	Yazdani *et al.*, 2009
Star anise (*Illicium verum*)	8	Yazdani *et al.*, 2009
Candle Bush (*Senna alata*)	5	Sule *et al.*, 2010
Ficus exasperata	39.81	Mbakwem-Aniebo *et al.*, 2012
Ocimum gratissimum	52.40	Mbakwem-Aniebo *et al.*, 2012
Polygonum aviculareae	100	Maoz and Neeman, 1998
Polygonum equistiforme	100	Maoz and Neeman, 1998
Cynodon dactylon	100	Maoz and Neeman, 1998
Inula viscosa	25	Maoz and Neeman, 1998

Prosopsis farcta	100	Maoz and Neeman, 1998
Amni visnaga	12.5	Maoz and Neeman, 1998
Salvia fruticosa	100	Maoz and Neeman, 1998
Celltis australis	100	Maoz and Neeman, 1998
Tamarix aphylla	100	Maoz and Neeman, 1998
Laurus nobilis	25	Maoz and Neeman, 1998

Analysing the tables, it´s possible to observe that the MIC values for the different plant extracts listed on both tables are higher than those obtained on this study (tables 3.2 and 3.3). Thus, although the natural composition is different, it´s possible to conclude that chitosan is a much more efficient natural antifungal agent against the two species of dermatophytes studied than other agents studied by several authors. However, it is important to highlight that these sensibility may be strain dependent, and strains used in this study are different from the studies reported, which can affect the results and justify the differences found.

For *Aspergillus* species, it wasn´t possible to determine the MIC values for any of the tested chitosan, because it would be necessary higher concentrations of chitosan, that are not feasible in a stable solution to be tested, due to chitosan's poor solubility and high viscosity for concentrations higher than 1.5% (in particular for high MW chitosan).

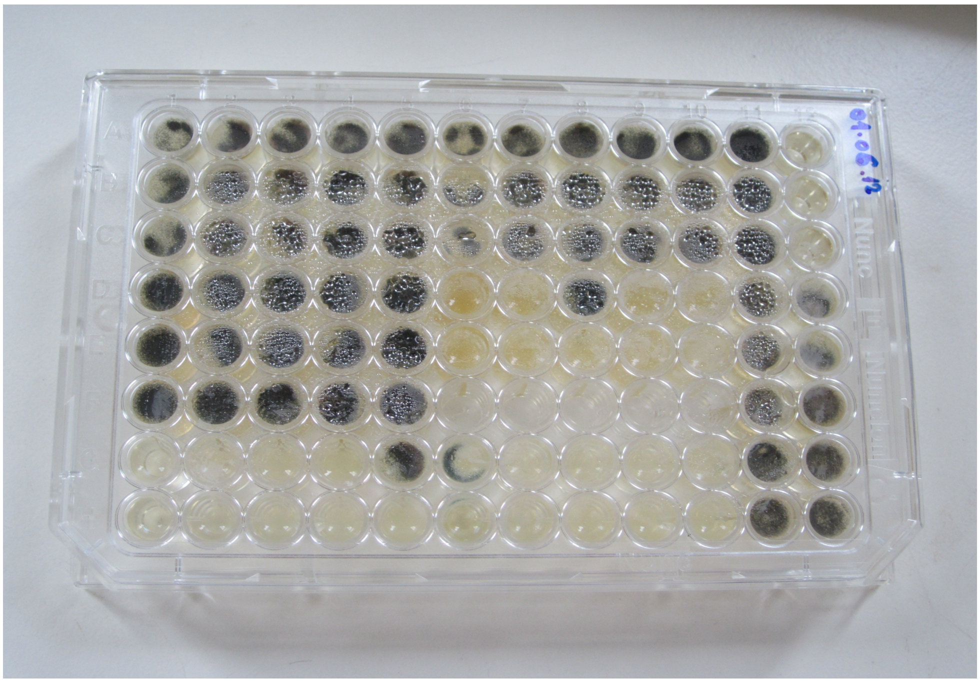


Fig 3.2. Photograph of microplate used to determine MICs. The tested fungus was *Aspergillus niger*. MICs could not be determined.

Some authors also studied the antifungal action of chitosan in some *Aspergillus* species. De Souza *et al.* (2013) reported in their study that chitosan MIC for *A. flavus* is above 4 g/L after 72 h of incubation. In the other hand, Yoonkyung *et al*. (2008) studied the antifungal action of low MW chitosan on *A. fumigatus* but were also unable to achieve a positive MIC value (> 2,5 mg/mL).

Other authors induced modifications on chitosan molecule and studied the antifungal action of these modified chitosans. Lam and Diep (2003) treated chitosan with radiation and studied the antifungal activity of this chitosan on some fungal species. For *A. fumigatus*, they obtained a MIC value of between 120 and 150 ppm depending on the radiation absorved by the chitosan's molecule. Mohamed and Fahmy (2012) study the antifungal action of a chitosan hydrogel on *A. fumigatus* and *A. niger.* They obtained a MIC value of 250 µg/mL for *A. fumigatus* and a MIC value of 125 µg/mL for *A. niger.*

Analysing the results of the mentioned studies, it´s possible to verify that, positive results for MIC values are achieved when chitosan was modified. When chitosan did not suffer any modification, no positive result for MIC value was obtained, which is also verified in this thesis.

The resistance of *Aspergillus* species to chtiosan can be due to:

1) the existence of chitosan on the cell wall of some *Aspergillus* species, and for that reason this polymer is less effective against fungi with chitosan in their cell walls (Limam, *et al.*, 2011).
2) their ability to degrade chitosan due to the production of chitosanases and others enzymes with chitinolytic activity (Palma-Guerrero *et al.*, 2007; Ashoub *et al.*, 2009).

3.1.2. Determination of Minimum fungicidal concentration (MFC)

Besides MICs determination, minimum fungicidal concentrations (MFC) were also determined for *T. rubrum* and *M. canis*. These results are listed in the table below:

Table 3.4 – Minimum fungicidal concentration of HMW, MMW and LMW chitosans determined for *T. rubrum* and *M. canis*.

Minimum fungicidal concentration (mg/mL)			
	High MW chitosan	**Medium MW chitosan**	**Low MW chitosan**
T. rubrum	1.3	1.6	2.2
M. canis	1.3	1.6	1.1

Comparing these results with MIC, is apparent that MFC values are the same as the MIC values for both fungi, so inhibitory concentration corresponds also to fungidal concentration.

Similarly to the approach performed for MICs' values, a comparison of chitosan's MFCs obtained in this work with MFCs values of other natural antifungal substances was done. Those MFCs data obtained on other studies are listed on following tables:

Table 3.5 – Minimum fungicidal concentration of some natural antifungals determined for *T. rubrum*.

T. rubrum		
Antifungal drug	**MFC (mg/mL)**	**Reference**
Chili (*Capsicum annuum*)	9.3	Vaijayanthimala *et al.*, 2004
Coffee (*Coffea arabica*)	9.3	Vaijayanthimala *et al.*, 2004
Cumin (*Cuminum cyminum*)	9.3	Vaijayanthimala *et al.*, 2004
Turmeric (*Curcuma longa*)	4.6	Vaijayanthimala *et al.*, 2004
Curry leaf (*Murraya koenigii*)	18.7	Vaijayanthimala *et al.*, 2004

Table 3.6 – Minimum fungicidal concentration of some natural antifungals determined for *M. canis*.

M. canis		
Antifungal drug	**MFC (mg/mL)**	**Reference**
Anise (*Pimpinella anisum*)	128	Yazdani *et al.*, 2009
Star anise (*Illicium verum*)	64	Yazdani *et al.*, 2009
Candle Bush (*Senna alata*)	5	Sule *et al.*, 2010

Once again, the MFC values for the different plant extracts listed on both tables are higher than those obtained on this study (tables 3.5 and 3.6). Thus, it´s possible to conclude that chitosan is a more efficient natural fungicidal agent against the two species of dermatophytes studied than other agents studied by several authors.

3.1.3. Conclusions

So far, and based on these results, it´s possible to conclude that chitosan has an antifungal action against *T.rubrum* and *M. canis*. This antifungal action is concentration dependent because, as said previously, at higher concentrations of chitosan no fungal growth was observed. However, unlike other studies (Li, *et al.*, 2008), the antifungal action of chitosan against dermatophytes is not molecular weight dependent.

For *Aspergillus* species, it was not possible to ascertain chitosan's MIC probably, because, these fungi has some resistance mechanisms against this compound.

MFC were also found for both dermatophyte fungi, corresponding to the same values of MICs.

Another conclusion we can draw from this study is that chitosan is a more efficient antifungal and fungicidal drug than other common natural drugs extracted from plants.

3.2. Inhibition of keratinolytic activity in hair model

Dermatophytes, as keratinolitic fungi, through the production of keratinases can degrade hair (or other keratinolizide tissues) and cause an infection called *tinea capitis*. Like all dermatophytosis, tinea capitis is contagious and requires prolonged treatment periods. In fact, some studies suggest that a 4-week treatment with terbinefine is insufficient for complete elimination of *Microsporum* infections (Devliotou-Panagiotidou and Koussidou-Eremondi, 2004). Therefore, chitosan as antifungal agent could make an alternative to this slow treatment. Additionally, if chitosan is not at least at minimum inhibitory concentrations, lower concentrations cannot completely inhibit the growth, but could inhibit some of the virulence factors like keratinolytic enzymes and prevent the infection.

Thus, in order to validate this hypothesis in this work, human hair was tentatively infected with *T. rubrum* and *M. canis* and the chitosan with high, medium and low MW was added previously to the infection. Some samples were collected after one, two and three weeks of incubation and submitted to scanning electron microscopy analysis. Purposes of this test were to see the morphological changes that occur when a hair is infected with dermatophytes and understand the differences between the samples treated and non-treated with chitosan during the time of the experience.

3.2.1. Analysis of hair by Scannig Electron Microscopy

As dermatophytes present different manifestations during the hair infection, it´s necessary to understand, at first glance, how hair fibber is composed. A hair fibber consists in three morphological regions. From inside to outside, these regions are: medulla, cortex and cuticle. The diameter of a hair fibber varies from 50 to 90 µm (Saengkaew, *et al.*, 2011). Human hair is mostly composed of fibrous α-keratin proteins.

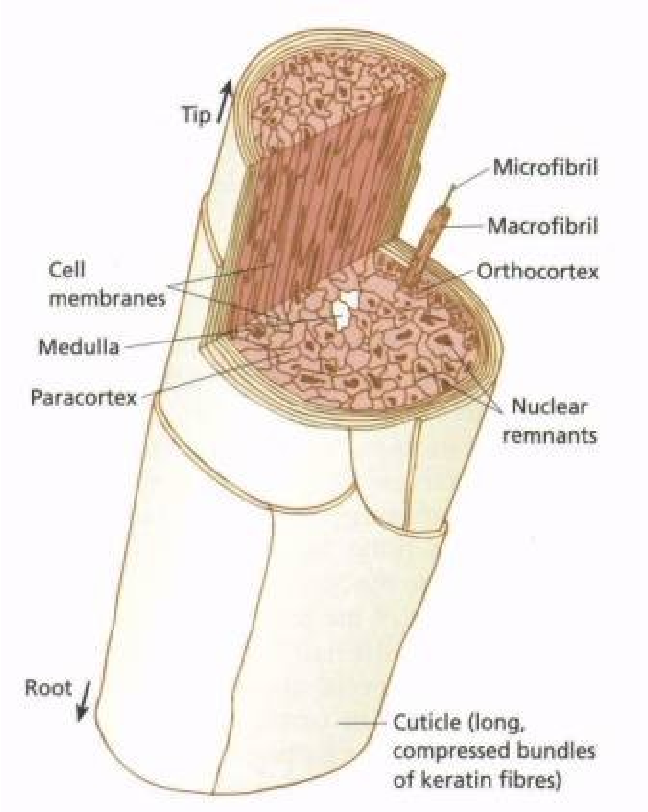
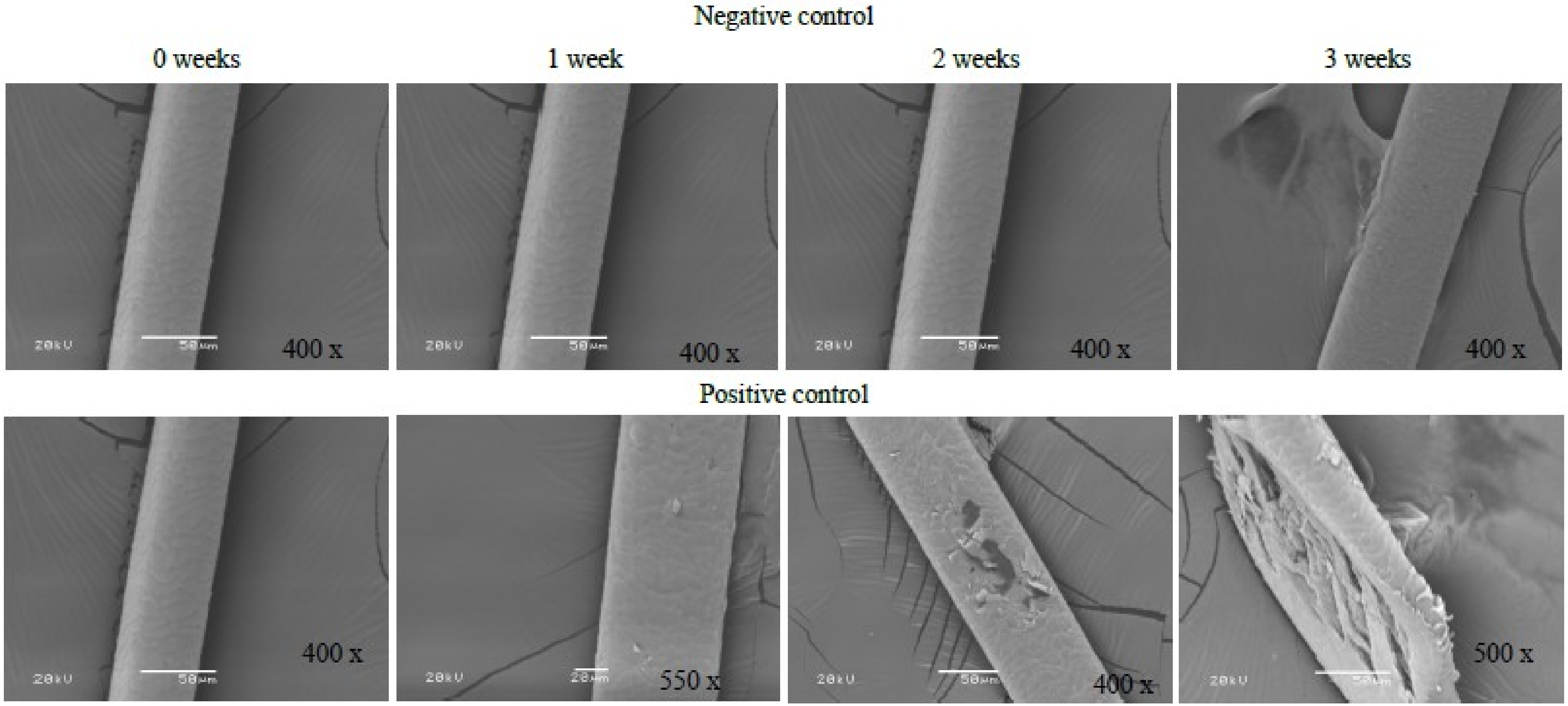


Fig 3.3. A scheme of human hair representing the different layers

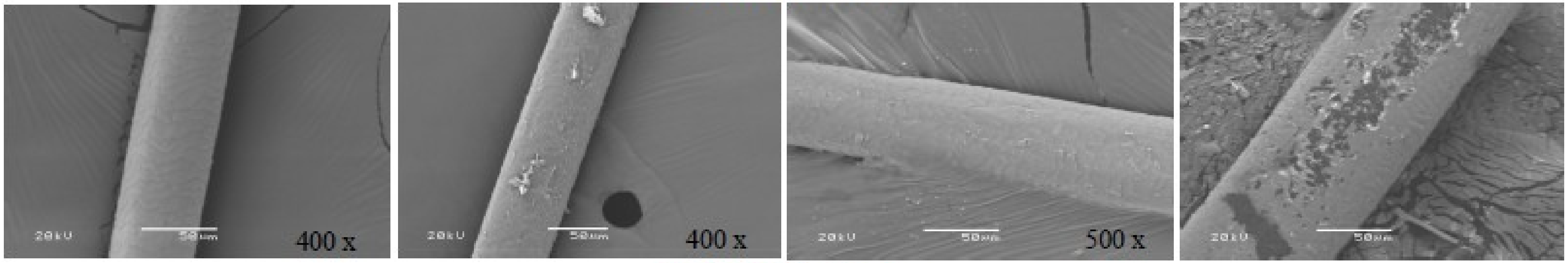
In figure 3.3 representing a human hair shows the different morphological regions of the hair. The sequence of degradation of hair structures depends on their degree of keratinization. So, in the cuticle, penetration of the hyphae below the scales is followed by invasion of non-keratinized cell membranes and the endocuticle with its many citoplasmatic residues. In the cortex, the cell membrane and cytoplasmatic residues are digested in the first place. Then, the destruction of the intermacrofibrillar matrix, the thin sheet and the microfibrils occurs. The most resistant part of the hair is the intermicrofibrillar matrix (microfibril), which is the last part to be degraded (Marchisio, 2000).

In the following figures, pictures of hairs infected with dermatophytes and treated with tested chitosans can be observed.

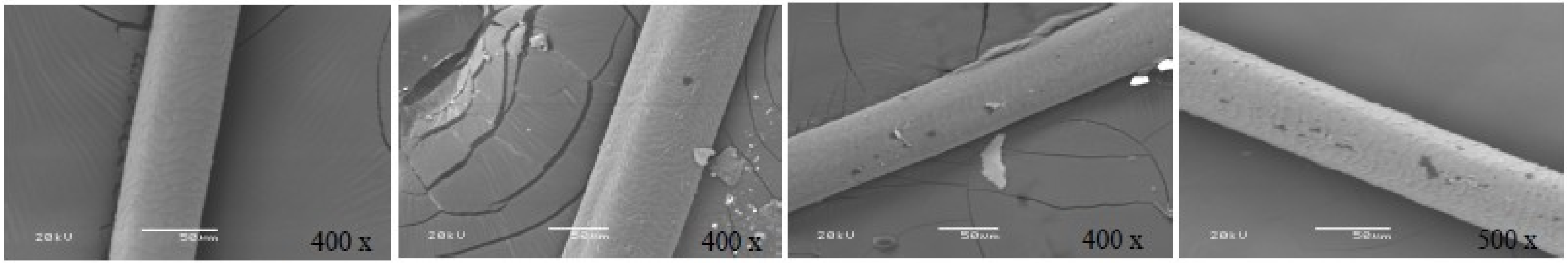
Fig 3.4. Scanning electron microscopy pictures of human hair. The hair was infected with *T. rubrum* and treated with HMW, MMW and LMW chitosans. Negative control (no infected hair) pictures and positive control (infected hair) pictures are also shown. Hair samples were analysed after 1, 2 and 3 weeks of incubation.



High MW chitosan



Medium MW chitosan



Low MW chitosan

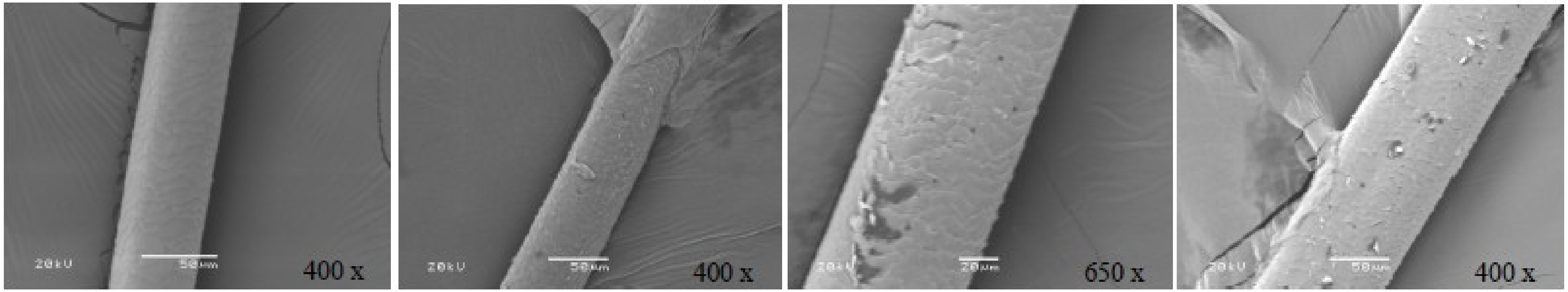
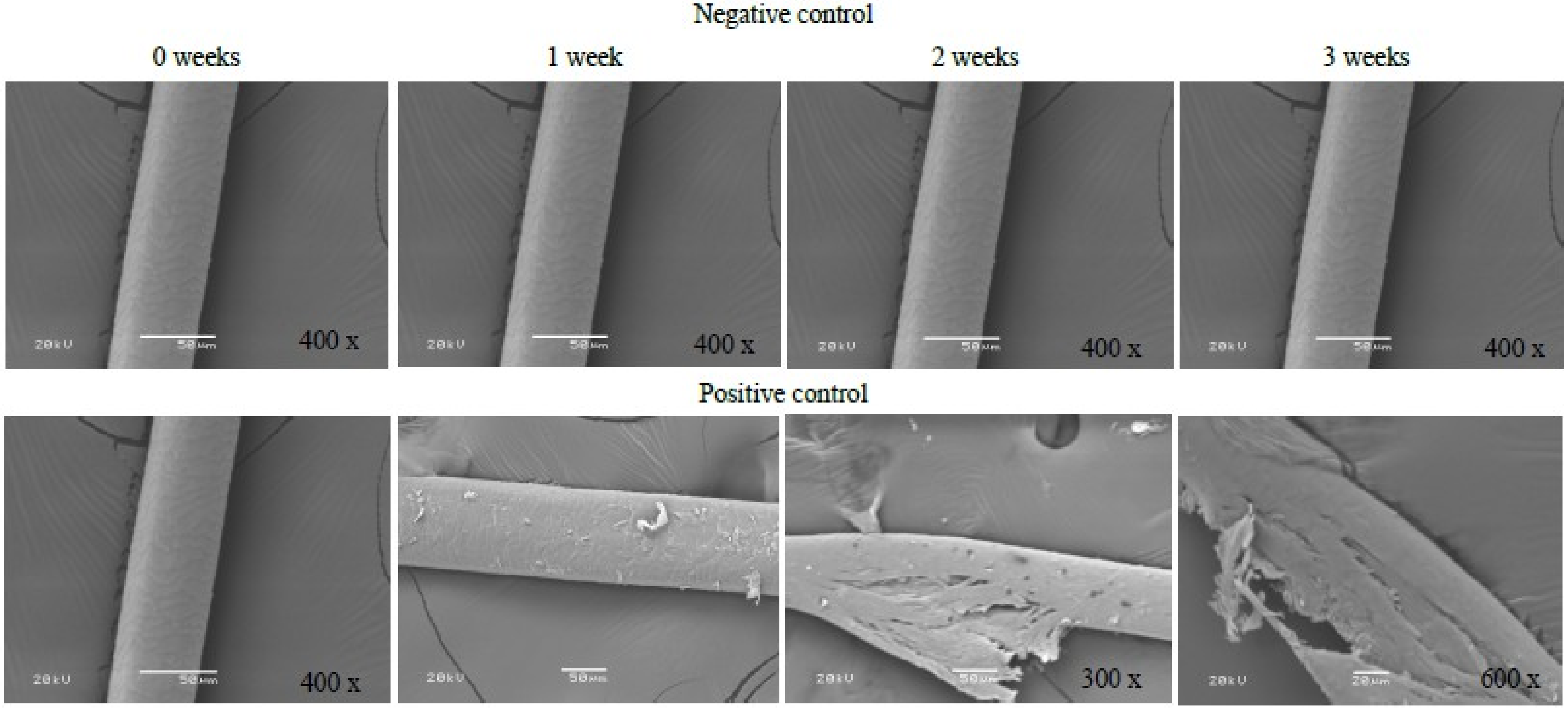
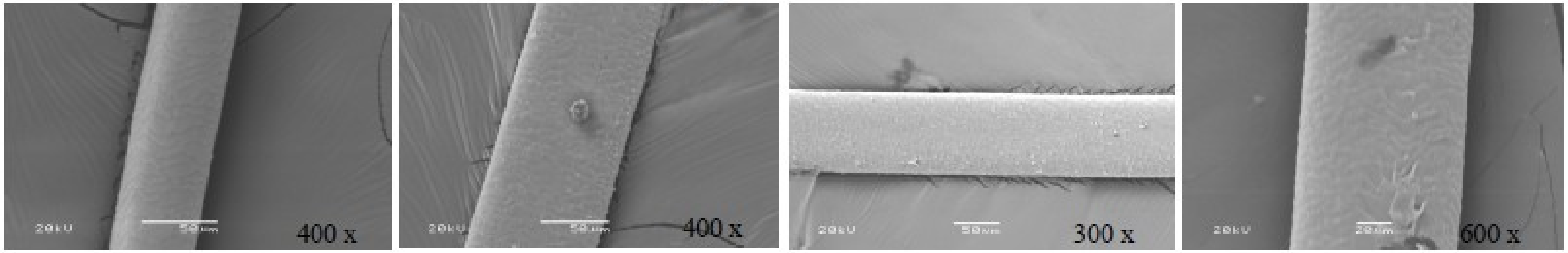


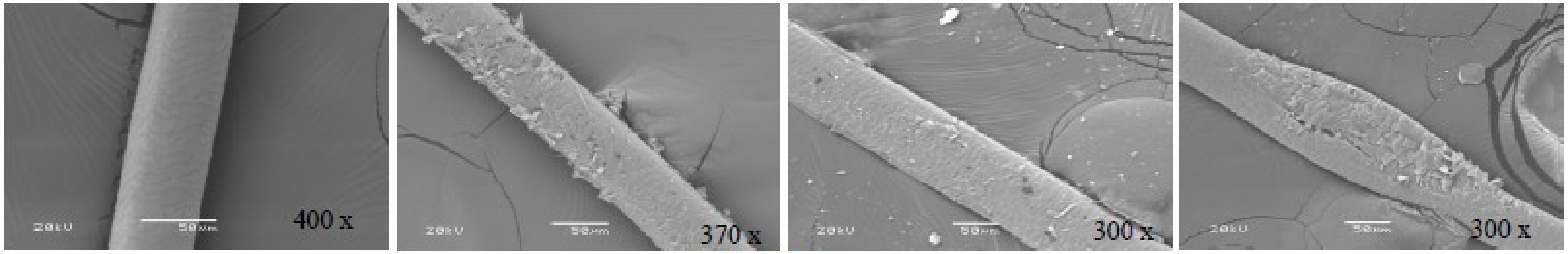
Fig 3.5. Scanning electron microscopy pictures of human hair. The hair was infected with M. canis and treated with HMW, MMW and LMW chitosans. Negative control (no infected hair) pictures and positive control (infected hair) pictures are also shown. Hair samples were analysed after 1, 2 and 3 weeks of incubation.



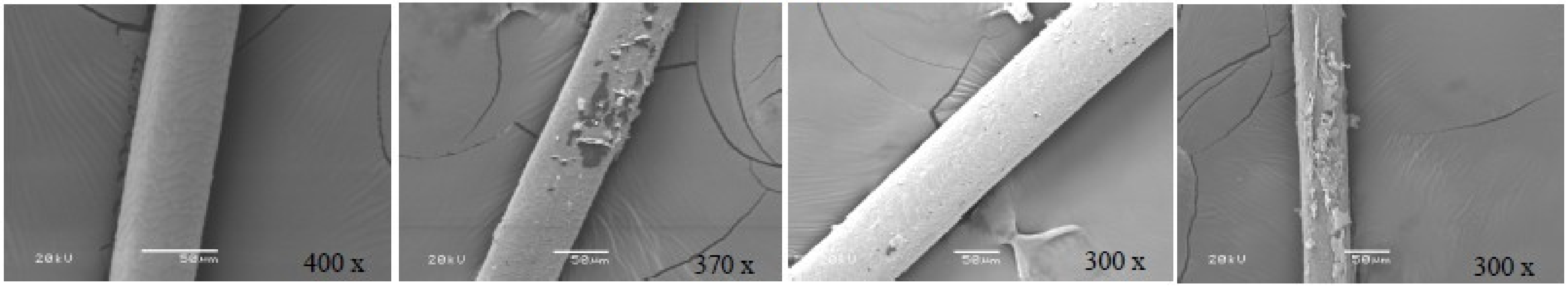
High MW chitosan



Medium MW chitosan



Low MW chitosan



In general when detached hairs are attacked by keratinolitic fungi, as dermatophytes, it's possible to visualize the following stages in hair surface:

- spores separated on the cuticular layer;
- abnormal pattern of the cuticular cells of the hair shaft when compared with negative control;
- cuticle cells of the hair were raised and they were peeled off;
- regularity of the cuticle was altered in some areas (Ozdemir *et al.*, 2010).

These stages of infection can be observed in the positive control pictures (fig. 3.3 and 3.4) which demonstrate the infection progress for each fungus:

T. rubrum

After one week of incubation, the hair still shows normal appearance because it is not possible to see any spores or hyphae. Damages caused by the fungus are minimal as the hair presents only a few abnormalities – some cuticular cells are partly raised and keratin seems to be a little bit degraded as the hair shows in the surface a structure similar to a hole.

In the second week of incubation, the regularity of the cuticle was altered in some areas and the hair seems to be bumpy.

Finally, after three weeks of incubation, the hair is completely destroyed. At this stage, although it´s possible to observe an abnormal pattern of the cuticular cells and modifications in the regularity of the cuticle, the most impressive is the destruction of the hair. The fungus was not only destroyed the in upper layers but infection progressed and destroyed the inner layers, too.

M. canis

Only after one week of incubation, the hair already showed visible damages since an abnormal pattern of the cuticular cells was already visible, when compared with the same stage of infection on *T. rubrum* infected hair.

In the second week of incubation, spores and hyphae were visible on the cuticular layer and the fungus was capable of destroy the upper layers of the hair.

At the end of the experimental time, the hair is much more destroyed confirming the infection progress.

These ultrastructural differences of the hair when infected with different dermatophytes could be explained by differences in keratin degradation. In fact, *T. rubum* cause flat erosions of the hair surface (Katiar and Kushwaha, 2012), while *M. canis* produces radial penetration (Marchisio, 2000).

Flat erosion is a gradual destruction of hair from the outside inwards by hyphae. Hyphae extend under the cuticular scales, lift up the cuticle and then digest the scales starting from the inner side. These hypahe may keep their normal appearance or dilate to form short branches and originate structures similar to the palm of a hand (Marchisio, 2000).

In the radial penetration, there is a random attack by specialized hyphae. They penetrate the hair perpendicularly to its surface. These structures are called boring hyphae and perforating organs (Marchisio, 2000). According Scott and Untereiner (2004), the production of perforating bodies is always accompanied by some degree of surface erosion.

These different behaviours during hair infection, explains the reason why the hair infected by *T. rubrum* seems to be less degraded, since flat erosion is a gradual form of hair destruction unlike radial penetration.

After a brief analysis of the differences in hair infection by the two studied dermatophytes, the effect of chitosan on these infections patterns is analysed.

T. rubrum

High MW chitosan treatment: After one week of incubation, in the hair treated with HMW chitosan is not apparent any degradation. The precipitates-like structures arranged in the uppermost layer of the hair do not seem to be fungal structures, but precipitated chitosan.
The same pattern is visible in the second week of incubation, although some fungal structures may be present too. Finally, after 3 weeks of incubation, the hair shows some evidence of damage on the surface, however very reduced in extension when compared with positive control. Additionally, despites the existence of some small "holes" in the hair surface, indicators of fungal activity, the most notorious anomalies seem to be chitosan depositions. These results demonstrate that the high MW chitosan delayed the infection signs possibly by the inhibition of keratinases production or their activity.

Medium MW chitosan treatment: After one week of incubation, the hair treated with medium MW chitosan looks normal and the only abnormality observed is a darker spot that seems to be a “hole”. The hair also presents a white structure that seems to be a chitosan deposit. In second week of the experience, the hair shows few more “holes” and very few cuticle cells from hair are raised. A fungal spore can also be observed. At last, after the third week of incubation, the hair exhibit more but only a slight degradation when compared with positive control for the same week. So once again, for this medium MW chitosan is also evident a reduction of keratinases activity or even their production.

Low MW chitosan treatment: In the first week of incubation, the hair presents a chitosan structure that resembles a protection film on the surface, but few chitosan precipitates are also apparent with almost no evidence. After two weeks of incubation, few holes are visible, with a slight abnormal pattern of the cuticular cells of the hair. At the end of experience, the hair shows some damage signs in particular some holes and some cells are raised and pelled but in a small extension; the existence of a chitosan precipitate is also evident. Despites some evidence of damage, the extension is also quite reduced when compared with equivalent period in the control, confirming also that chitosan also inhibits keratinases production or their activity.

M. canis

High MW chitosan treatment: At the end of the first week of the experiment (figure 3.5), the hair showed a normal appearance, i.e. it does not show any sign of fungal degradation only a fungal spore can be observed on the cuticle. After two weeks of incubation, similar result was observed; the hair has a normal aspect with some chitosan precipitates on the surface. At the last week of the experiment, chitosan precipitates were also evident, but, this time, a shadow as a hole can be observed suggesting slight fungal activity. These reduced activity showed the strong effect of the high MW chitosan upon the inhibition of *M. canis* queratinolytic activity upon the hair.

Medium MW chitosan treatment: After one week of incubation (see figure 3.5), despite the treatment with chitosan, *M. canis* was able to form fungal structures (mainly hyphae). Alongside with the presence of chitosan precipitates, cuticle cells of the hair were slightly raised and few of them were pelled off. Very few holes were also visible. In this pattern these holes are probably perforating organs. After two weeks of incubation, the hair presented perforating organs that are larger in size. Chitosan precipitates could also be observed. After three weeks of incubation, the hair is quite damaged, the regularity of the cuticle was altered in some areas and the fungus started to invade inner layers. However, the effect of *M. canis* infection was quite delayed and reduced compared with the control.

Low MW chitosan treatment: In the first week of incubation, the hair did not show any sign of fungal infection. It is possible to observe (figure 3.5) in the uppermost layers chitosan precipitates. After two weeks of infection, fungal spores are visible on the cuticle and very few perforating organs could also be observed, as well as chitosan precipitates. At the end of the experiment, the hair seems to be covered by a chitosan's film with precipitates that may protect it from the fungal invasion.

3.2.2. Conclusions

Analysis of SEM pictures, allowed the following conclusions:

- *M. canis* is capable of degrading hair faster than *T. rubum* since after two weeks of incubation it was able to destroy the upper layers of the hair whereas *T. rubrum* only achieved this after three weeks.
- It is evident that chitosan can efficiently reduce the effects of a dermatophyte infection in human hair, since lower damage upon hair was observed
- High MW chitosan showed to be the most efficient in preventing hair degradation for both fungal infections. Hair treated with this type of chitosan presented less perforation organs when infected with *M. canis* and showed less flat erosions when infected with *T. rubrum*.
- Chitosan demonstrated a protective effect. In some cases, chitosan (mainly low MW chitosan) showed the formation of film-type covering the hair that may protect it from fungal action.

3.3. Inhibition of keratinolytic activity by keratinase enzymatic activity and keratin degradation tests

Chitosan can inhibit different stages in fungal development such as mycelial growth, sporulation, spore viability and germination and production of virulence factors (Li *et al.*, 2008).

Scanning electron microscopic photographs of hair infected with dermatophytes and treated with chitosan showed significant differences between treated and not-treated hair. This suggests that chitosan, even when used at sub-MIC concentrations, has an antifungal effect. This effect as previously explained, possibly occurs by inhibition of one of most important virulence factors, their keratinases. In order to better understand and explore this effect, keratinase enzymatic activity and keratin degradation tests were also performed.

3.3.1. Keratinase enzimatic activity tests

Dermatohytes are the most widespread group of hyphomycetes that can degrade keratin completely and have a great interest for scientists due to their pathological importance (Awasthi and Kushwaha, 2011).

Keratins are insoluble proteins which are resistant to degradation by common proteolytic enzymes such as trypsin, pepsin and papain. This is true because these proteins have a high degree of cross-linking by disulphide bonds, hydrogen bonding and hydrophobic interactions (Saber *et al.*, 2010).

Keratinases are enzymes that catalyse the hydrolysis of keratins. These enzymes are key in fungal invasion of skin and skin formations (Awasthi and Kushwaha, 2011).

After analysis of the results of previous experiment, it became clear (by scanning electron microscope) that chitosan could affect keratin degradation since significant differences between treated and non-treated samples of infected hair can be observed. However, those results didn’t allow establishing the specific activity of chitosan on hair protection, or on keratinases activity.

So, an attempt to understand the effect of different chitosans on enzymatic activity of keratinases was performed. For this purpose the colorimetric method described by some authors (Matikevičienė *et al.*, 2009; Awasthi and Kushwaha, 2011) to determine the enzymatic activity of keratinases was carried out with some adaptations.

However, and despite all the efforts, this method proved to be ineffective in the determination of keratinase activity using solutions with chitosan, because it was verified contradictory values of keratinase activity for these solutions. This fact could be explained by the existence of a precipitate in the bottom of the test tube that is probably a result of chitosan interaction (highly reactive) with the azure dye used in the substrate of enzymatic reaction.

In fact, chitosan has been used as a chelating agent and even as an adsorbent agent to remove dyes, because it possesses amino and hydroxyl groups, that serve as active sites (Ngah, *et al.*, 2011).

So, in order to understand the chitosan activity on keratinases, the standard method of determination of keratinase activity was tentatively replaced by an enzymatic test using protease K. Results are depicted in figure 3.6.

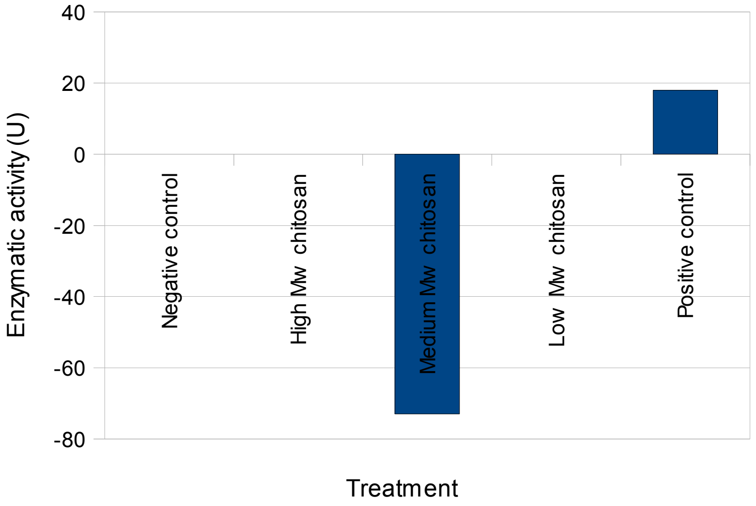


Fig 3.6. Enzymatic activity of proteinase K under HMW, MMW and LMW chitosans' action. Positive control corresponds to absence of chitosan

Figure 3.6 displays that keratin-azure samples digested with protease K shows a value of enzymatic activity of 18 U. Contrasting positive controls, samples digested with protease K and treated with chitosan (high, medium and low MW) did not exhibit any keratinase activity. This suggests that chitosan can interfere with dermatophytes proteolytic activity.

With this test, results allow us to understand chitosan’s mode of action. Since in this test we fix an amount of proteinase K, the absence of enzymatic activity shows that this polymer leads to the inhibition of the enzyme activity, possibly by interaction of the chitosan with the protein itself. Additionally, based on these results it is possible to hypothesise that in the results observed in infected hair (high content of produced keratinases) the chitosan did not prevent keratinase production, but possibly inactivated enzymatic activity.

So, it seems clear that chitosan's action is maybe expressed in inhibition of protein´s action. In fact, as mentioned by Kunert (2000), dermatophytes' proteases are often inhibited by chelating agents because they sequester Ca^{2+} ions. These ions are frequently bound by serine proteases and they are essential for proteases activity because they increase their activity and stability.

3.3.2. Keratin degradation test

On this test, keratin-azure was used as the only protein source in the medium. Keratin-azure is a blue compound that become colourless upon cleavage (Sharma and Rajak, 2003).

When keratin-azure is degraded by a keratinophilic fungus, the blue dye is released into the culture medium. The migration of the azure dye is a measure of the keratin degradative ability of the keratinolitic fungi.

When chitosan is added to the culture medium is expected to lower the azure dye realease into the medium. In the pictures 3.7 a) and 3.7 b) illustrate the dye release after 4 weeks for all the treatments tested.

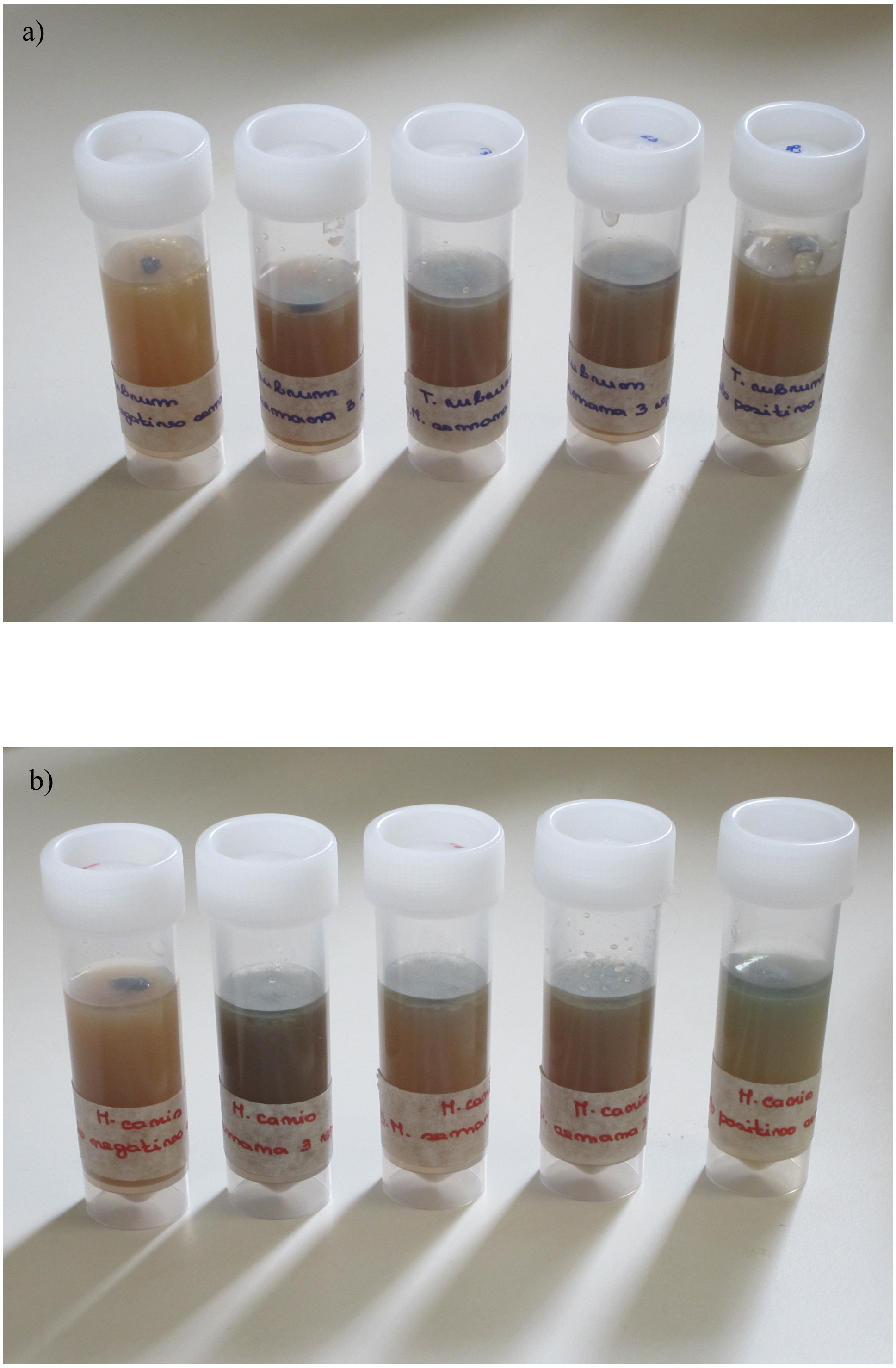


Fig 3.7. Azure dye release after 4 weeks of incubation with *T. rubrum* (a) and *M. canis* (b). From left to right: negative control, HMW chitosan treatment, MMW chitosan treatment, LMW chitosan treatment and positive control.

T. rubrum didn´t show dye release in none of the treatments with chitosan (figure 3.8) and present a weak release in positive control tube (figure 3.9).

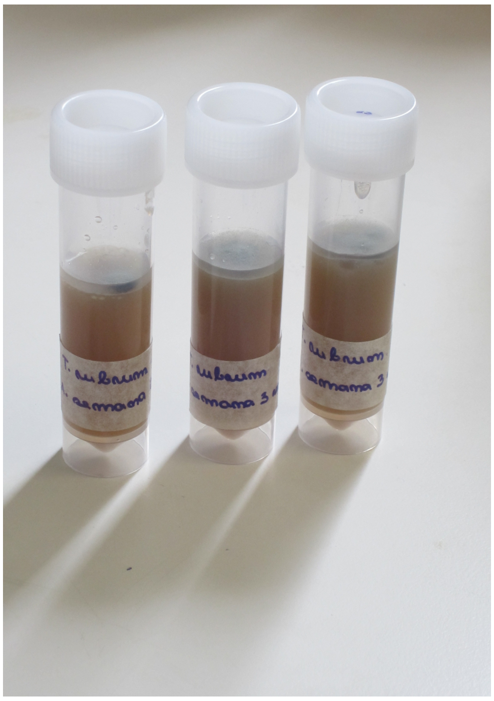


Fig 3.8. Azure dye release after 4 weeks of incubation with *T. rubrum* in HMW chitosan treatment, MMW chitosan treatment and LMW chitosan treatment.

This occurs because this fungus does not produce perforating bodies. According Scott and Untereiner (2004), *T. rubrum* cannot degrade keratin-azure and release the azure dye into the culture medium.

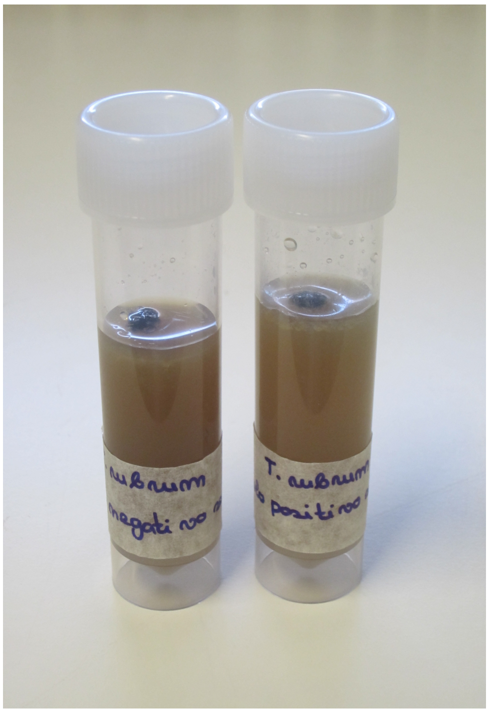


Fig 3.9. Azure dye release after 4 weeks of incubation with *T. rubrum* in negative control (left) and positive control (right).

However, *M. canis* showed dye release in the positive control tube because it produces perforating bodies. According Scott and Untereiner (2004), the production of perforating bodies is always accompanied by some surface erosion, and for that reason *M. canis* can degrade keratin-azure and release the azure dye into the culture medium. *M. canis* presented dye release in chitosan-containing tubes, but this was weaker than that observed on the positive control. No dye release or fungal growth was observed in the negative controls.

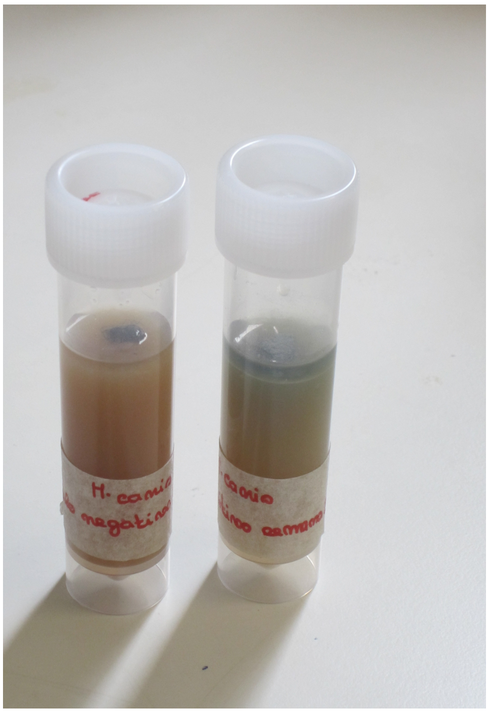


Fig 3.10. Azure dye release after 4 weeks of incubation caused by *M. canis* in negative control (left) and positive control (right).

Scott and Untereiner (2004) also mentioned that density of growth did not appear to be correlated with the degree of dye release because, as these authors verified in their study, some fungi, like *Amauroascus purpureus*, cause dye release and does not present any visible growth. In this thesis, this fact is also verified because *T. rubrum* presented substancial growth but the dye release was very weak or even absent. *M. canis*, unlike *T. rubrum*, showed abundant growth but it also present dye release.

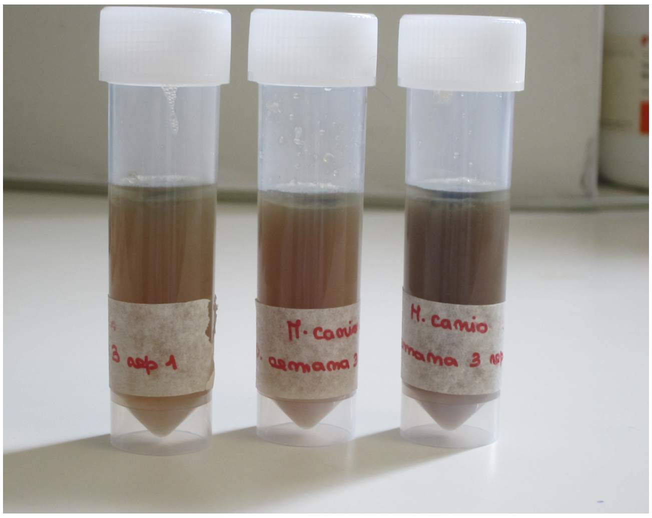
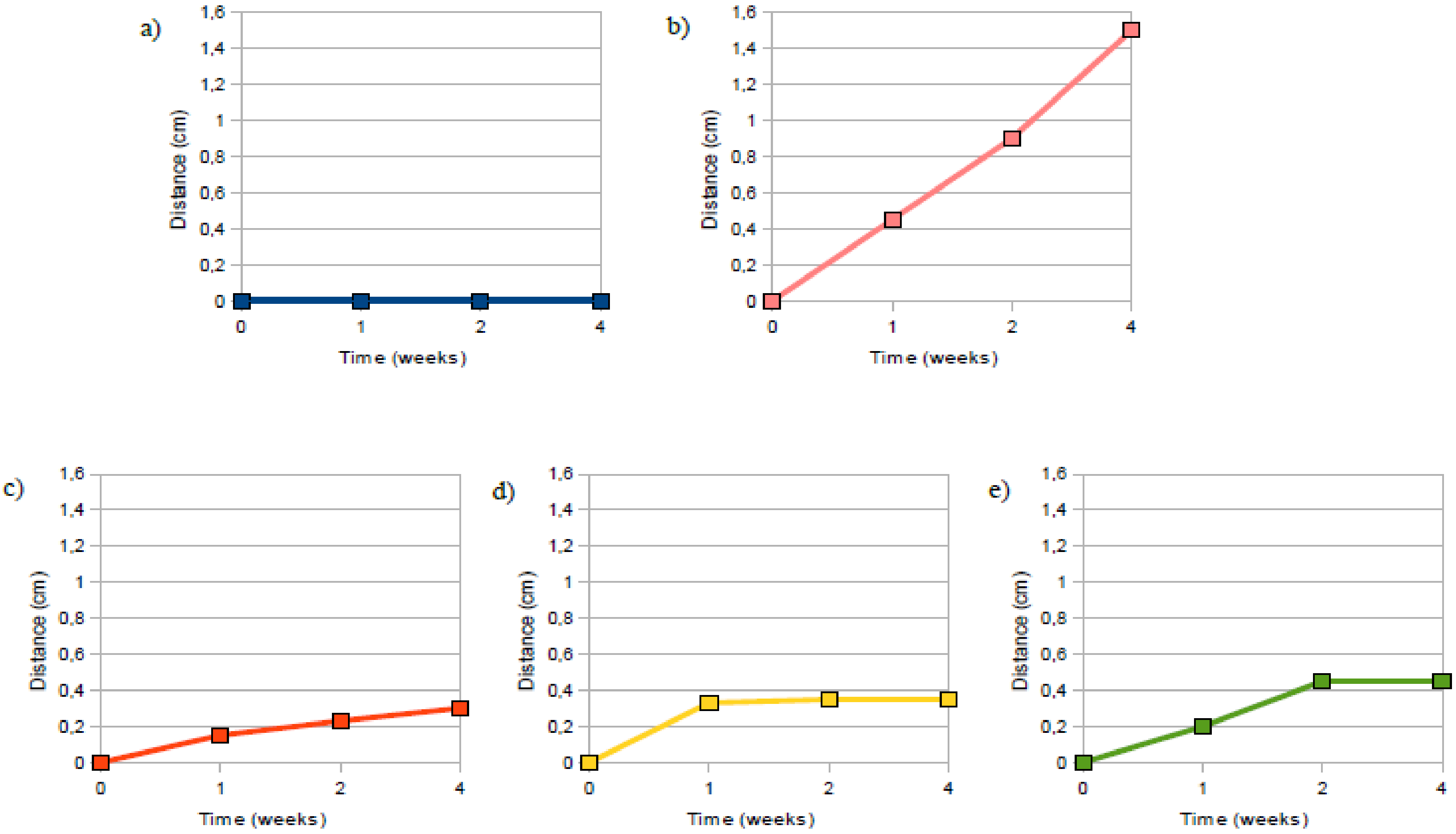


Fig 3.11. Azure dye release after 4 weeks of incubation with *M. canis* in HMW chitosan treatment, MMW chitosan treatment and LMW chitosan treatment.

Because *M. canis* showed a different dye release when exposed to the various chitosans, the distance travelled by the dye released from the keratin was measured after one, two and four weeks of incubation. The results are organized in the graphs below:

Fig 3.12. Azure dye release during 4 weeks for *M. canis:* a) negative control, b) positive control, c) HMW chitosan treatment, d) MMW chitosan treatment and e) LMW chitosan treatment.



Analysing the positive control, it is possible to verify an increase in the migration of azure dye throughout incubation time that corresponds to a linear relation (R^2= 0,99) between the incubation time and the displacement of azure dye. The azure dye release is probably dependent on the growth and maturity of the fungus. Thus, at longer incubation times, more azure dye will be released.

Figure 3.10 shows that chitosan interferes with keratin degradation. It is clear that all the chitosans tested were able to prevent keratin degration because, as can be seen in the graphics obtained from samples treated with chitosan, all the distance travelled by dye release at the end of the experiment time is approximately the same that is observed in the positive control after one week of incubation.

In fact, the displacement of azure dye release with high MW chitosan treatment only slightly increased during the incubation time up to a maximum of 0,3 cm after four weeks of incubation, that corresponds to a linear relation (R^2= 0,96) between the incubation time and the displacement of azure dye.

On the other hand, for the medium MW chitosan, the migration of azure dye increase up to 0,35 cm, after one week of incubation (at similar rate of the control) but remained constant until the end of incubation time.

Regarding low MW chitosan treatment, the migration of azure dye increase linearly up to 0,45 cm by two weeks of incubation (presenting similar values as control for one week) but remained constant until the end of incubation time.

Although it is apparent that medium MW chitosan seems to be the less efficient chitosan in preventing keratin degradation, since the initial rate is higher, in fact all the chitosans led to similar and significant inhibition of migration (corresponding to inhibition of keratin degradation) after 4 weeks of incubation when compared with control, corresponding to a reduction ranging 70-77% of azure dye displacement. So, all MW chitosans seem to be equally efficient in preventing keratin degradation by *M. canis*.

3.3.3. Keratin degradation test – microscopic analysis

In order to verify the existence of a relationship between the analytical data and the events at a microscopic level, keratin-azure samples of each treatment were observed by scanning electron microscopy and on an optical microscope.

Pictures were obtained for both fungi (*T. rubrum* and *M. canis*).

Figure 3.11 shows a keratin-azure fibre that was not infected with the fungus or treated with chitosan.

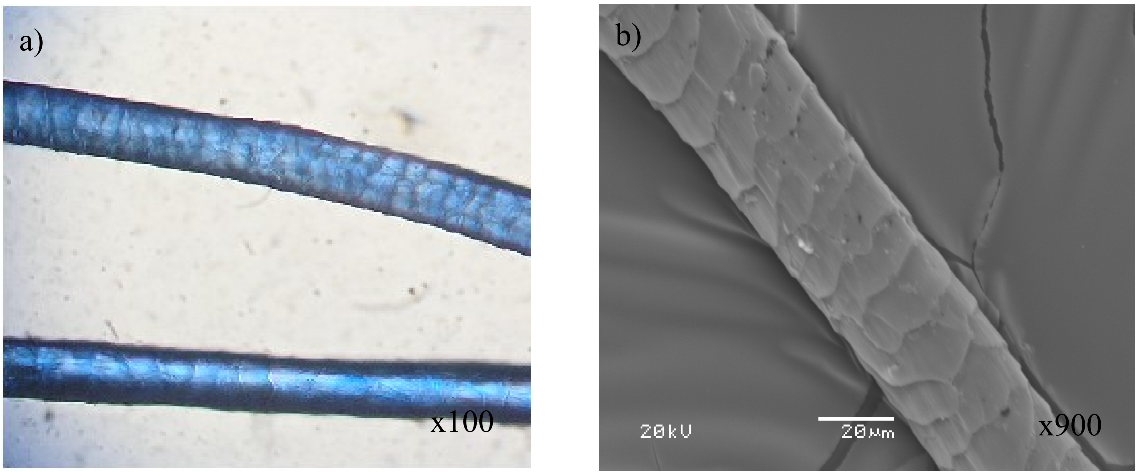
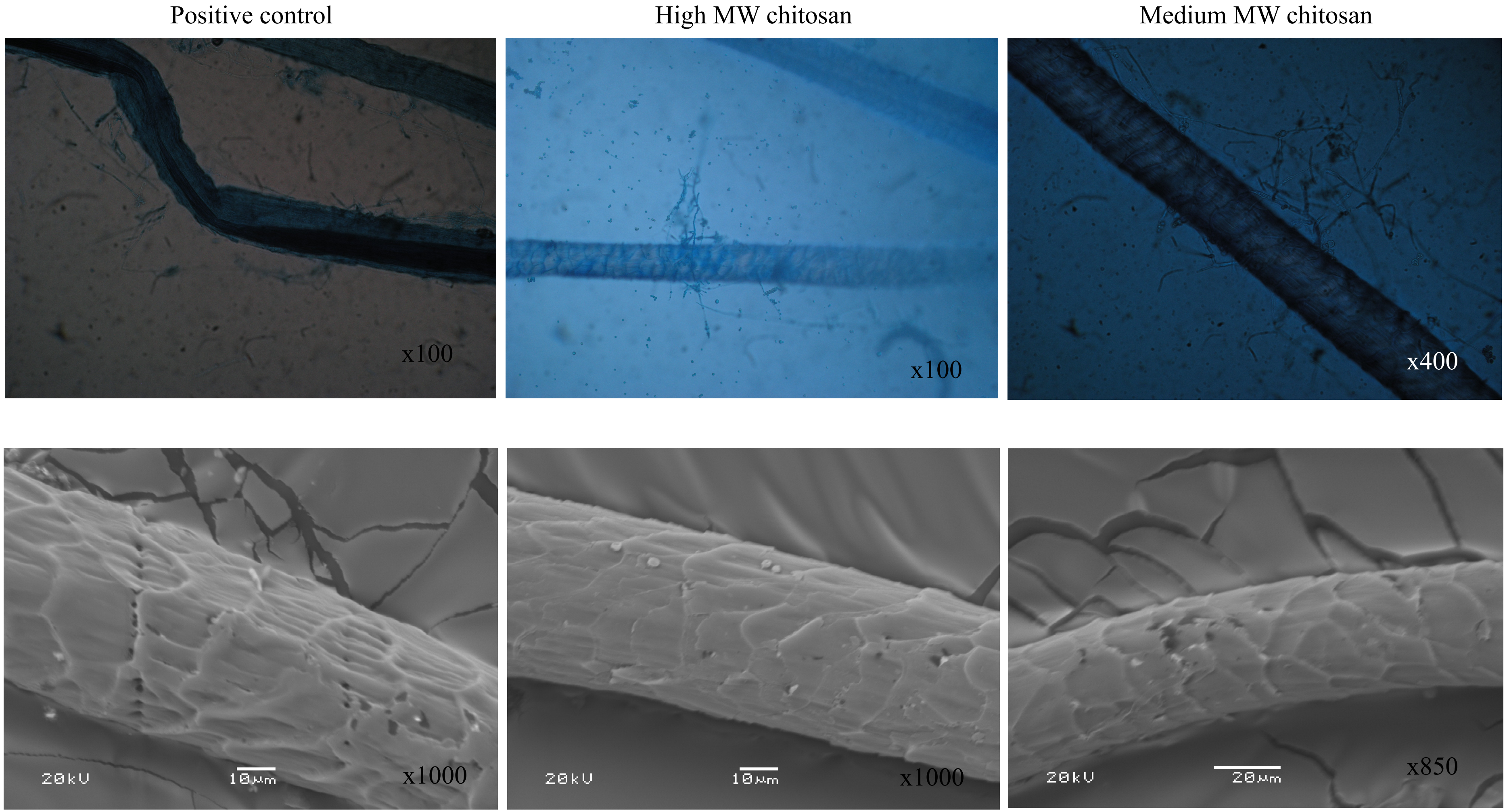


Fig 3.13. Keratin-azure: optic microscopy (a) and scanning electron micoscopy pictures (b).

The following figures (3.14 and 3.15) show keratin-azure threads infected with both fungi and treated with chitosan. For *T. rubrum*, high MW chitosan and medium MW chitosan are shown, whereas for *M. canis* only medium MW chitosan is shown. Positive control images are also dipicted for both fungi.

Fig 3.14. Optical and scanning electron microscopy pictures of keratin-azure. Keratin-azure was infected with *T. rubrum* and treated with HMW and MMW chitosans. Samples were collected after four weeks of incubation.

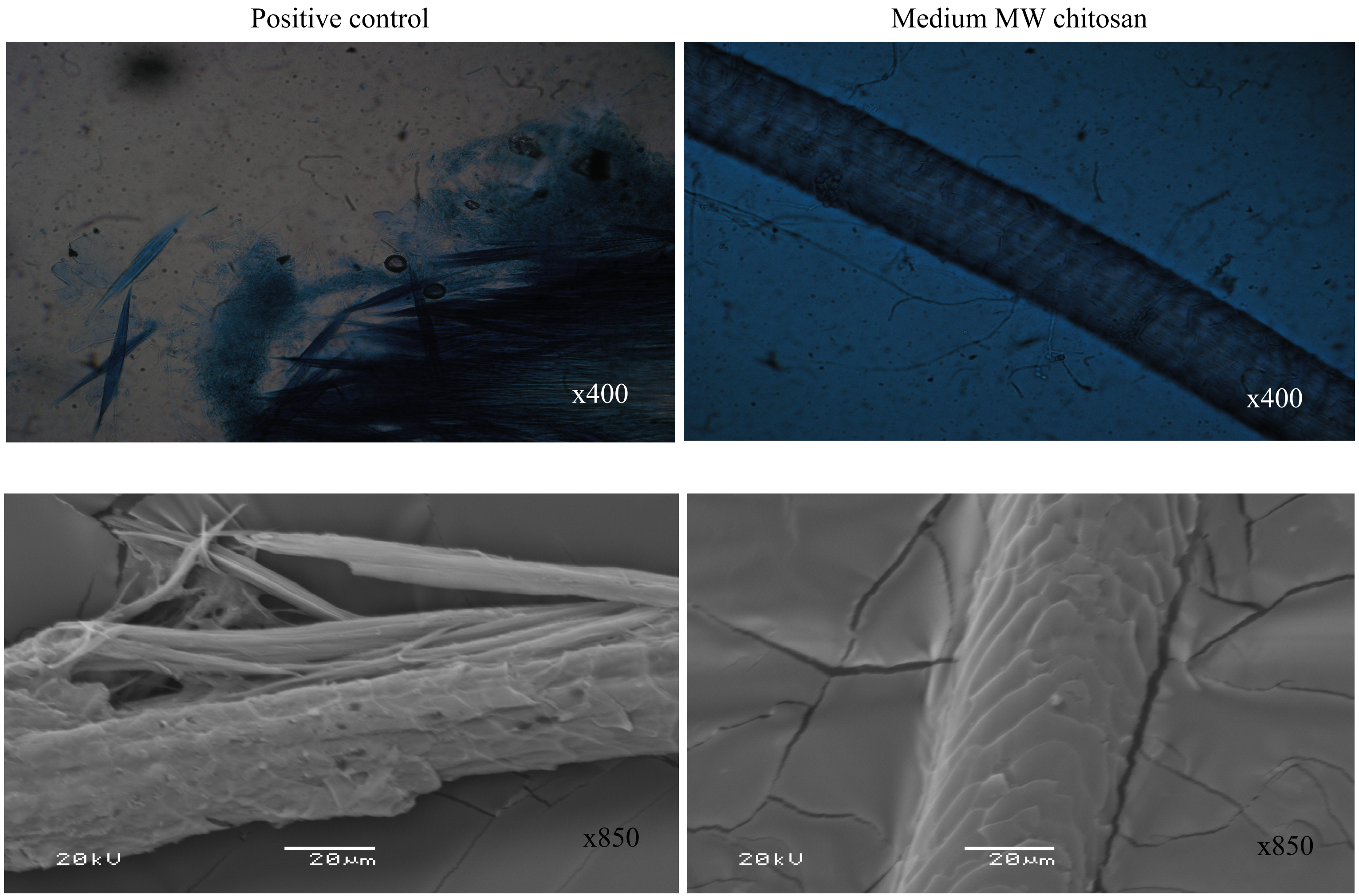


Positive control

High MW chitosan

Medium MW chitosan

Fig 3.15. Optical and scanning electron microscopy pictures of keratin-azure. Keratin-azure was infected with *M. canis* and treated with MMW chitosan. Samples were collected after four weeks of incubation.



Positive control

Medium MW chitosan

T. rubrum

Positive control: The picture obtained by optical microscopy shows fungal hyphae around the keratin-azure fibre but no signs of fungal destruction are evident. However, analysis of scanning electron microscope images allow to observe some holes in the fibre surface. No other sign of fungal infection is visible. This could possibly be explained by the inability of *T. rubrum* to produce perforating bodies, thus causing more keratin degradation.

High MW chitosan treatment: The picture obtained with optical microscope shows fungal hyphae and spores. Nevertheless, the fungus seems to organize itself around and on the top of the keratin-azure and not causing the fiber's degradation. This is even more remarkable in scanning electron microscopy image as it´s possible to observe some fungal spores on the keratin-azure fiber. Aditionally, the number of holes caused by the fungus is lower than that observed on positive control.

Medium MW chitosan treatment: Once again, in the optical microscope image is possible to see fungal hyphae and spores. And, once more, the fungal seems to arrange itself on the top and around the keratin-azure fiber not causing any visible damage. This is observed on scanning electron microscopy image as keratin-azure appears to be intact as the picture is very similar to the negative control.

M. canis

Positive control: In the first image it is possible to observe that keratin-azure is quite different from what we can see on the negative control picture. Furthermore, large masses of fungal mycelium can be observed. The differences in keratin-azure aspect are even more obvious when we look at on scanning electron microscopy image and we can observe the severe damage of the fibre. Additionally, it also possible to observe some holes in the fiber of keratin-azure surface.

Medium MW chitosan treatment: In the optical microscopy image it is possible to observe some fungal hyphae and spores. They are located on top and around the keratin-azure, but no sign of degradation is visible. In the scanning electron microscopy image, an intact keratin-azure fiber with fungal spores on the top was observed.

3.3.4. Conclusions

Dermatohytes are fungi that have the ability of growing on keratinized substrates. Through the production of proteolytic enzymes, mainly keratinases, they degrade these subtracts. It is known that chitosan acts as a chelating agent, rendering metals, trace elements or essential nutrients that become unavailable for the fungus to grow at the normal rate (Li *et al.*, 2008). However, it is important to understand if chitosan has an effect in preventing keratinases' action.

The enzymatic activity test done with protease K revealed that chitosan has, in fact, an important activity in preventing proteases action. Furthermore, chitosan could prevent keratin degradation by *M. canis* and *T. rubrum*. The keratin degradation test showed that when chitosan is present in culture media, the dye release is lower than when it is not present. Although out all the chitosan molecules tested are efficient, the medium MW chitosan seems to be the less efficient chitosan in preventing keratin degradation.

A microscopic study of the surface of keratin-azure both in the presence and absence of chitosan, led to the same conclusion. In positive control pictures, we saw a degraded keratin-azure fiber. This degradation was lower in *T. rubum* infection because this fungus does not produce perforating bodies. For both fungal infections, fibers treated with chitosan seemed to be intact despite the existence of fungal structures around them.

4. General conclusions

The main objective of this work was to evaluate the antifungal activity of selected chitosans upon some dermatophytes and some selected species of *Aspergillus* sp. Also, the antifungal action of chitosan upon dermatophytes in a keratinased matrix was evaluated.

The study of antifungal activity of chitosan showed that chitosan possesses a relevant antifungal action against *T.rubrum* and *M. canis,* presenting MIC ranging 1.1 and 2.2 mg/mL, depending on the MW of chitosan. The antifungal action is concentration dependent as expected. The antifungal action of chitosan against dermatophytes is MW dependent but depends on the type of fungi. For *Aspergillus* species, it was not possible to find the chitosan's MICs, because, although chitosan can affect their growth, in the range of concentrations used the total inhibition was not achieved, revealing much lower susceptibility of this fungi to chitosan. Minimum fungicida concentrations were also obtained for both dermatophytes, showing same values as MICs. We can also conclude from this study that chitosan is a more efficient antifungal compound than other natural drugs, open new opportunities for dermatophytosis treatment.

As dermatophytes are responsible for hair, skin and nail infections, is crucial to understand if chitosan even at sub-MIC concentration exert any effect on fungi activity, in particular on queratinolitic substracts. In this study, human hair was used as substract. The analysis of SEM images let us conclude that chitosan seems to have a protective effect of the hair when this was infected with dermatophytes, since it was evident lower damage of hair when fungi grew in the present of chitosan. High MW chitosan seems to be the most efficient in preventing hair degradation for both fungal infections − hairs treated with this chitosan present less perforation organs when infected with *M. canis* and show less flat erosion when infected with *T. rubrum.* In some situations, chitosan (mainly low MW chitosan) is able of producing a kind of film that protects the hair from fungal action.

In order dermatophytes can grow on keratinized substracts, they have to produce proteolytic enzymes, mainly keratinases, to degrade these substracts. In this work the effect of chitosan on enzymatic activity was tested using protease K; in fact, an important activity in preventing proteases action was observed, possibly because it acts as a chelating agent rendering Ca^{2+} ions, which are important for protease K action and, probably, for others proteases action.

The effect of chitosan on keratin degradation by *M. canis* and *T. rubrum*, was also studied by keratin-azure test. The results showed that the dye release is reduced when chitosan is present in culture media; medium MW chitosan showed to be the less efficient chitosan in preventing keratin degradation.

A microscopic analysis of keratin-azure surface when chitosan was present in the culture media corroborated the conclusion. The positive control images exhibited a degraded keratin-azure fibre, however the degradation was reduced in *T. rubum* infection because this fungus does not produce perforating bodies. For both fungal infections, keratin-azure surface treated with chitosan showed to be intact despite the existence of fungal structures around them.

Overall, this study revealed that chitosan showed relevant antifungal activity against dermatophytes, proving also to interfere with dermatophytes infection mechanisms. So, these chitosan properties together with its biocompatibility open good prospects to chitosan as an alternative for the usual tinea treatments.

5. Future work

This work allowed us to take some new and relevant conclusions concerning chitosan antifungal activity upon dermatophytes growth and activity. However, several questions remained unanswered representing unexplored possibilities for future work:

1) Extend the experiments cover in present study to others species of dermatophytes;

2) Use skin and nail as new model fungal substrates and analyse chitosan's activity on these substrates;

3) Test other methods to study keratinolytic activity;

4) Study chitosan effect on others virulence factors relevant on dermatophytes, namely lypases, estereases, phosphatases, amylases and other proteases.

5) Develop a product incorporating chitosan (eg. nail varnish, cream or solution) that ensure the bioavailability of this polymer and allow to be applied easily by patients, demonstrating efficiency and stability

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