# PANOMYCOCIN-INCORPORATED CHITOSAN-TPP NANOPARTICLES: PREPARATION, CHARACTERIZATION AND *IN VITRO* DETERMINATION OF ANTIFUNGAL ACTIVITY AGAINST HUMAN DERMATOPHYTES

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## ABSTRACT

PANOMYCOCIN-INCORPORATED CHITOSAN-TPP NANOPARTICLES: PREPARATION, CHARACTERIZATION AND *IN VITRO* DETERMINATION OF ANTIFUNGAL ACTIVITY AGAINST HUMAN DERMATOPHYTES

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There is an increase in the incidence of superficial fungal infections. Especially, immuno-compromised patients, diabetic patients, obese and people having resistancy against a group of antifungal drugs are at great risk. In these patient groups the infection can become chronic and affect the deeper and living layers of the skin and can be life threatening if not treated effectively. However, drugs which are currently used in the therapy of dermatophyte infections are becoming less effective due to the serious side effects and resistance developed by the pathogens. Thus, there is a continuing need for new classes of antifungal compounds that have potent antifungal activity, improved safety and low tendency to elicit resistance. Among the different approaches, the discoveries of naturally occuring antifungal proteins with little or no toxicity towards mammalian cells are attracting increasing attention. Within the naturally occuring antifungal proteins the yeast killer proteins which are produced and secreted into the environment by yeast strains with a killer phenotype represent promising candidates as potential antifungal agents in the medical field. Among the most effective killer toxins, the K5 type toxin has been purified and characterized for the first time in our laboratory and named as Panomycocin. Panomycocin is an exo-β-1,3 glucanase with a molecular mass of 49 kDa. It kills the sensitive cells by hydrolysing the β-1,3-glucans which are vital polymers for the integrity of the fungal cell wall. In following studies we have formulated Panomycocin with chitosan-TPP nanoparticles, which are used for the encapsulation of therapeutic proteins and accepted as effective non-toxic carrier system and characterized the nanoparticles in terms of surface morphology, particle size, zeta potential, interactions between chitosan, TPP and Panomycocin and *in vitro* release of Panomycocin from chitosan-TPP nanoparticles. Lastly, we have determined *in vitro* antifungal activity of Panomycocin-incorporated chitosan-TPP nanoparticles against common human dermatophytes. All tested dermatophyte strains were found to be susceptible to

Panomycocin-loaded CS-TPP NPs. MIC-0 range for *Tricophyton* species was found as 1-2 mg/ml and MIC-0 for *Microsporum gypseum* was found as 1.5 mg/ml.

This study will enable the development of a new, highly selective antifungal drug for the topical treatment of human superficial infections. In addition to providing health benefits, Panomycocin will contribute to the economy as it is produced naturally.

**Keywords:** Panomycocin, dermatophytes, superficial skin infections, chitosan-TPP nanoparticles.

# ÖZ

PANOMYCOCİN İÇEREN KİTOSAN-TPP NANOPARTİKÜLLERİ: PREPARASYONU, KARAKTERİZASYONU VE İNSAN DERMATOFİTLERİNE KARŞI *İN VİTRO* ANTİFUNGAL AKTİVİTESİNİN BELİRLENMESİ

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Günümüzde yüzeyel mantar/maya enfeksiyonlarının yaygınlığında çok büyük bir artış görülmektedir. İmmün sistemi baskılanmış hastalar, diyabet hastaları, immün sistemi zayıf, şişman ve belli antifungal ilaçlara direnç kazanmış kişiler büyük risk altındadır. Bu hasta gruplarında enfeksiyon kronik hale dönüşebilir ve derinin derin ve canlı kısımlarını da etkiler ve etkili bir şekilde tedavi edilemezse hayati sorunlara neden olabilir. Ancak tedavide kullanılan antifungal ajanlar ciddi yan etkilere neden olup, etkinlikleri yüzeyel deri enfeksiyonlarına neden olan patojenlerin bu terapilere direnç kazanmalarından dolayı azalmaktadır. Bu nedenlerle yüzeyel mantar enfeksiyonlarının tedavisinde yan etkisi minimal düzeyde olan, patojen mikroorganizmaların direnç geliştiremeyeceği yeni ajanların keşfedilmesi büyük önem kazanmıştır. Özellikle doğal antifungal proteinlerin araştırılması ve bu proteinlerin terapide kullanılması öngörülmüş ve desteklenmiştir. Antifungal etkiye sahip olan doğal proteinler arasında bazı maya suşları tarafından üretilen öldürücü proteinler günümüzde bir çok araştırmanın konusu olmuştur. Bu antifungal proteinlerin en etkililerinden biri olan K5 tipi maya toksik proteini tarafımızdan ilk defa saf halde izole edilmiş ve Panomycocin olarak adlandırılmıştır. Panomycocin moleküler kütlesi 49 kDa olan bir ekzo-β-1,3-glukanazdır ve fungal hücrelerin hücre duvarlarının yaşamsal polimeri olan ekzo-β-1,3- glukanları hidrolize ederek hücrelerin patlayarak ölmesine neden olur. Takip eden çalışmalarımızda Panomycocin'in terapötik proteinlerin enkapsüle edilmesi için sıkça kullanılan ve toksik etkisi bulunmayan etkili bir taşıyıcı sistem olan kitosan-TPP nanopartikülleri ile formülasyonu ve yüzey morfolojisi, partikül boyutu, zeta potansiyeli, kitosan, TPP ve Panomycocin'in etkileşimleri ve Panomycocin'in kitosan-TPP nanopartiküllerinden *in vitro* salımı bakımından karakterizasyonları yapılmıştır. Son olarak, Panomycocin içeren kitosan-TPP nanopartiküllerinin insanda yüzeyel deri enfeksiyonlarına neden olan yaygın dermatofit suşlarıüzerindeki in vitro antifungal aktivitesi belirlenmiştir. Bu çalışma sonucunda insanda yüzeyel deri enfeksiyonlarına neden olan dermatofitlere karşı

yeni, doğal ve yüksek seçiciliği olan güçlü fungisidal etkide bir proteinin ilk defa topikal bir preparat haline getirilmesi mümkün olacaktır. Panomycocin'in preparat olarak geliştirilmesi, hasta sağlığına getireceği faydaların yanında doğal yollarla elde edilmesinden dolayı ekonomik katkı sağlayacaktır. Panomycocin içeren chitosan-TPP nanopartiküllerin test edilen tüm dermatofit suşları üzerinde etkili olduğu gözlemlenmiştir. *Tricophyton* suşları için MIC-0 değeri 1-2 mg/ml aralığında, *Microsporum* gypseum için ise 1.5 mg/ml olarak bulunmuştur.

**Anahtar Kelimeler:** Panomycocin, dermatofitler, yüzeyel deri enfeksiyonları, kitosan-TPP nanopartikülleri.

***To my mother and grandmother,***

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| | |
|---|---|
| AE | Association efficiency |
| APS | Ammonium persulfate |
| BSA | Bovine serum albumin |
| CS | Chitosan |
| DD | Deacetylation degree |
| FTIR | Fourier transform infrared spectroscopy |
| LC | Loading capacity |
| kDa | Kilo dalton |
| kV | Kilo volt |
| MIC | Minimum inhibition concentration |
| MW | Molecular weight |
| NCYC | National Collection of Yeast Cultures |
| NP | Nanoparticle |
| PDA | Potato dextrose agar |
| SDS | Sodium dodecyl sulfate |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| SEM | Scanning electron microscopy |
| TEMED | Tetramethylethylenediamine |
| TPP | Tripolyphosphate |
| YEPD | Yeast extract peptone dextrose |

# CHAPTER 1

# INTRODUCTION

Dermatophytes are pathogenic filamentous fungi which cause superficialfungal infections termed as “dermatophytosis” (tineas or ringworm) which is one of the most frequent form of infections worldwide. The prevalence of dematophytosis has risen in the last decades that according to the World Health Organization (WHO), almost 25% of the world populationwas affected by dermatophytes and the incidence continues to increase. Dermatophytes are able to invade the stratum corneum layer of the epidermis and keratinized tissues, such as skin, nail, and hair of humans and animals and rarely invade the living tissue. They utilize keratinous substrates as nutrient sources (e.g., carbon, nitrogen and sulphur) during skin, hair, and nail infection by producing keratinase. There are about 40 species of dermatophytes belonging to three imperfect generad,namely *Epidermophyton*, *Microsporum* and *Trichophyton*. Dermatophytes are also classified as anthropophilic, zoophilic and geophilic species based on their habitat such as humans, animals and soil, respectively. Anthropophilic dermatophytes are associated primarily with humans and in some cases infect animals while zoophilic dermatophytes primarily infect animals and are transmitted to humans after direct contact with the involved animal, and geophilic dermatophytes are mainly associated with keratinous tissues of nonliving animals such as hair, feathers, horns, and hooves as after dissociating from living animals and are transmitted to humans from soil [1-4].

Generally, anthropophilic species has been more adapted to the human host than zoophilic species and characterised by relatively low inflammatory activity and a chronic infection of slow progressiondue to an immunological arrangement existing between the fungus and its human host and they represent about 70% of infections on humans, while zoophilic species cause about 30% of human dermatophytosis[3,5].

## 1.1 Epidemiology of Dermatophyte Infections

Dermatophytes exist in different regions of the world depending on climatic, geologic and social conditions with a high variability in the frequency of particular species.In developed countries, main risk factors are increasing migration and tourism, possession of pets, public sports facilities, the increasing number of patients having diabetes mellitus and vascular disease. Also, familiar disposition, foot trauma and cigarette smoking are known to increase possibility of dermatophytosis.

Dermatophytes flourish at temperatures of around 25–28 °C so in tropical and subtropical countries such as Africa and certain areas in Asia such as India, superficial fungal infections are more frequent and more distinctive due to favorable climatic conditions. Also, since socio-economic statuses of developing countries are worse than in Europe and the Americas, the prevalence of dermatophytosis is greater due to poor hygiene conditions, more skin contact between humans and close vicinity to animals due to crowded living conditions, while there are more problems concerningdisesase diagnosis and treatments. Moreover, in Africa, an important ratio of humans has HIV infections which weakens the immune systems so that favour fungal infections [3-5].Epidemiology of dermatophyte species is summarized in the Table 1.1.

**Table 1.1** Epidemiology of dermatophyte species with their ecological classification and host preference [2].

| Anthrophilic species (area of endemicity) | Zoophilic species (typical host) | Geophilic species |
|---|---|---|
| *E. floccosum* | *M. canis* (cat, dog) | *E. stockdaleae* |
| *M. audouinii* (Africa) | *M. equinum* (horse) | *M. amazonicum* |
| *M. ferrugineum* (East Asia, East Europe) | *M. gallinae* (fowl) | *Microsporum* anamorph of *A. cookiellum* |
| *T. concentricum* (Southeast Asia, Melanesia, Amazon area, Central America, Mexico) | *M. persicolor* (vole) | *M. boullardii* |
| *T. gourvilii* (Central Africa) | *T. equinum* (horse) | *M. cookei* |
| *T. kanei* | *T. mentagrophytes* (two sibling species and variants; rodents, rabbit, hedgehog) | *M. gypseum* (complex of three species) |
| *T. megninii* (Portugal, Sardinia) | *T. sarkisorii* (Bactrian camel) | *M. nanum* |
| *T. mentagrophytes* (complex of two species) | *T. simii* (monkey, fowl) | *M. praecox* |
| *T. raubitschekii* (Asia, Africa, Mediterranean) | *T. verrucosum* (cattle, sheep, dromedary) | *M. racemosum* |
| *T. rubrum* | | *M. ripariae* |
| *T. schoenleinii* | | *M. vanbreuseghemii* |
| *T. soudanense* (Subsaharan Africa) | | *T. ajelloi* |
| *T. tonsurans* | | *T. flavescens* |
| *T. violaceum* (North Africa, Middle East, Mediterranean) | | *T. gloriae, T. longifusum* |
| *T. yaoundei* (Central Africa) | | *T. phaseoliforme, T. terrestre* (complex of three species), *T. vanbreuseghemii* |

*T. rubrum* is the representative species of anthropophilic dermatophytes that is isolated from majority of clinical cases of tinea pedis (feet), tinea unguium (nails), tinea corporis (body) and tinea cruris (groin region).*T. mentagrophytesvar. interdigitale*, *T. tonsurans*,*E. floccosum*, *M. canis* or *M. gypseum* are rarely isolated as etiologic agents from patients with tinea [6-8].

### 1.2 Clinical Manifestations of Dermatophyte Infections

Dermatophyte infections are also named as tinea infections which are the fungal infections of skin. The clinical manifestations of dermatophytosis are; (i)tinea capitis (scalp); (ii) tinea barbae (beard and mustache); (iii) tinea corporis (arms, legs and trunk); (iv) tinea cruris (groin); (v) tinea manuum (hand); (vi) tinea pedis (feet); and (vii) tinea unguium (nails). The dominant causative agents of tinea infections and their clinical manifestations are given in Table 1.2 [1,2,5].

**Table 1.2** Common tinea infections and their causative agents [1].

| Type of tinea infections | Common species |
|---|---|
| Tinea capitis | *Tricophyton violaceum*<br>*T. tonsurans*<br>*Microsporum canis*<br>*M. gypseum* |
| Tinea corporis | *Trichophyton rubrum*<br>*T. tonsurans*<br>*M. canis*<br>*T. verrucosum*<br>*M. gypseum* |
| Tinea cruris | *T. rubrum*<br>*Epidermophyton floccosum*<br>*T. mentagrophytes var intergiditale* |
| Tinea pedis | *T. rubrum*<br>*T. mentagrophytes var intergiditale*<br>*E. floccosum* |
| Tinea manuum | *T. rubrum* |
| Tinea unguium | *T. rubrum*<br>*T. mentagrophytes var intergiditale* |

Symptoms of dermatophytosis can be seen as mild or severe depending on body location of infection, the host's immunologic condition and reactions to the metabolic products of the dermatophyte, the virulence of the infecting strain or species, and environmental factors. Skin infections generally have circular, erythematous and pruritic lesions (Figure 1.1) [3].

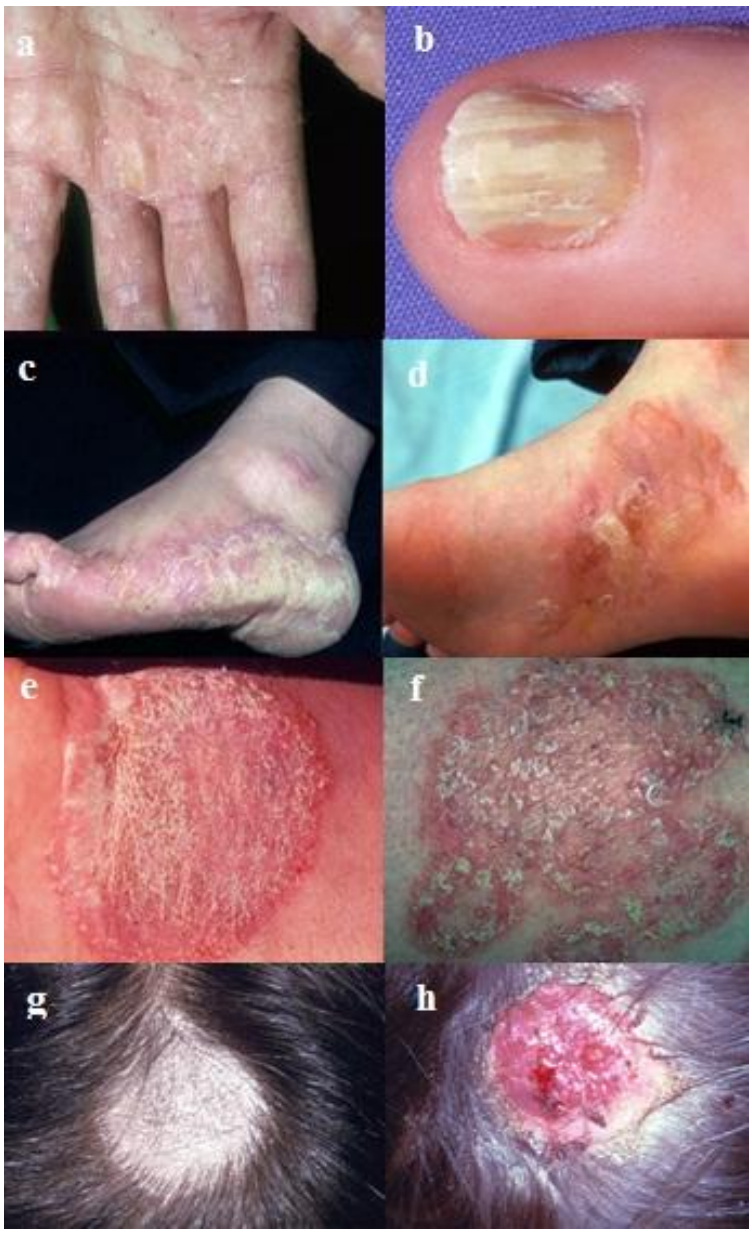



**Figure 1.1** Clinical manifestations of(a)Tinea manuum, (b) Tinea unguium , (c,d) Tinea pedis, (e,f)Tinea corporis, (g,h)Tinea capitis.

### 1.3 Risk Factors for Dermatophyte Infections

Since the fungi cannot penetrate the deeper tissues of healthy individuals because of the host defense responses, they are generally restricted to the cutaneous and the non-living corneum layers of epidermis preferring safe areas for their growth to hide from host defense mechanisms. However, these fungi can be invasive in individuals with compromised immune system and dermatophytes may invade deeper cutaneous and subcutaneous tissues [3,8].

Major risk factors responsible for the dramatic rise of dermatophytosis include prolonged and extensive use of broad-spectrum or indiscriminate antibiotics, the development of secondary resistance of pathogenic fungi to conventional drugs, immunosuppressive infections or diseases such as AIDS, leukemia, cancer and bone marrow and organ transplant patients, the use of antineoplastic agents, long-term use of corticoids and invasive medical procedures [9-11].

### 1.4 Antifungal Agents Used to Treat Dermatophyte Infections

Generally, antifungal agents can be classified based on their mechanism of action in pathogenic fungi (Figure 1.2) and they are administered by either systemically or topically depending on the fungal infection.

Azoles, polyenes, allylamines and morpholines target cell membrane, more specifically ergosterol which is the major cell membrane sterol of many pathogenic fungiand essential for membrane fluidity, asymmetry and integrity. They exert their action by inhibiting 14α-demethylase (lanosterol demethylase) in ergosterol biosynthetic pathway that is necessary for the conversion of lanosterol to ergosterol; binding to ergosterol which causes formation of porin channels and leakage of intracellular contents thereby causes loss of transmembrane potential and impaired cellular function; and inhibiting ergosterol biosynthesis by inhibiting squalene monooxygenaseas a result conversion of squalene to squalene epoxide, which is a precursor to lanosterol in the ergosterol biosynthesis, respectively.Echinocandinsexert their action by binding and inhibiting β-1,3-D-glucan synthase enzyme complex responsible for synthesis of cell wall β-1,3-D-glucan polysaccharides, the vital structural components of the cell wall in several common fungal pathogens.As a result of their action, fungal cells cannot maintain its shape and rigidity due to loss of glucans, leading to osmotic stress and eventually fungal cell lysis, especially in rapidly growing cells. Pyrimidine analoguesinterferewith pyrimidine metabolism which impairsDNA,RNA and protein synthesis. They are selectively taken up into the fungal cell and then deaminated to cytostatic 5-fluorouracil (5-FU) by cytosine deaminase and then it is converted into 5-fluorouridylic acid, which is phosphorylated by uracil phosphoribosyl transferase which inhibits thymidylate synthase. 5-FU is incorporated into RNA and causes RNA miscoding, premature chain termination and disruption of protein synthesis.5-Fluorouracil also is converted to 5-fluorodeoxyuridine monophosphate, which is a potent inhibitor of thymidylate synthase. Thymidylate synthase is involved in DNA synthesis and the nuclear division process and inhibition of this enzyme subsequently causes disruption of DNA synthesis.Mitotic inhibitorsbind to tubulin and interferes with microtubule formation [12-16].

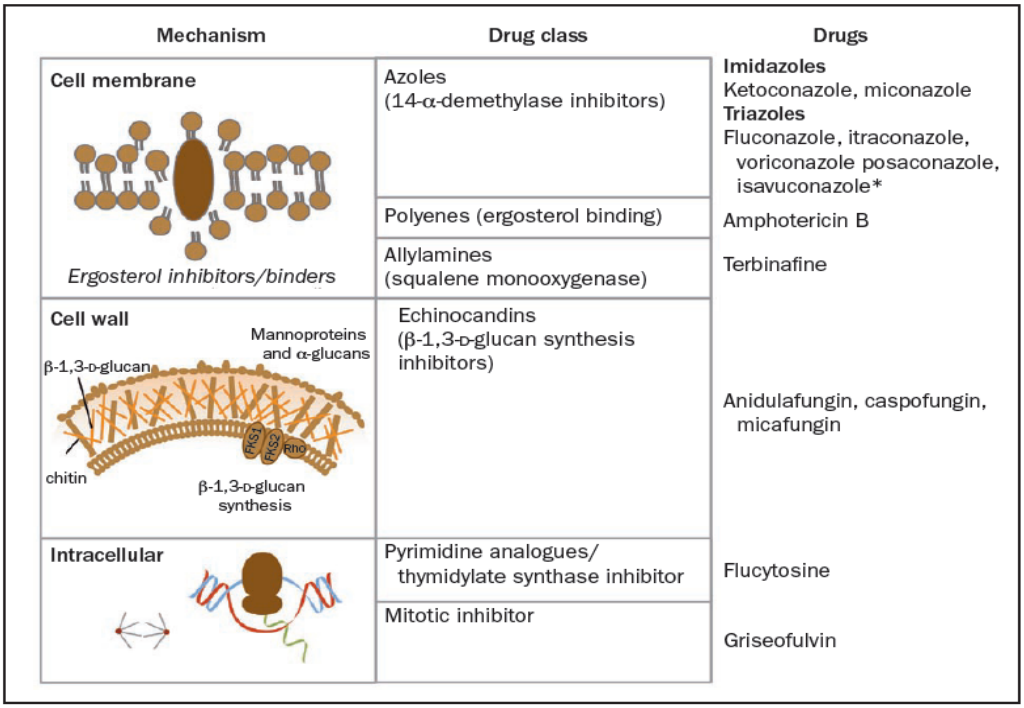



**Figure 1.2** Mechanisms of currently used antifungal agents andtheir cellular targets [14].

### 1.5 Antifungal Drug Resistance of Dermatophytes

Treatment of fungal infections is highly challenging task and one of the main reasons is the antifungal resistance which is insensitivity of a pathogenic fungus to an antifungal agent leading to failure of antifungal therapy. There are primary (intrinsic), secondary (acquired) and clinical antifungal resistances. Primary resistance naturally exists among particular fungi species without prior exposure to antifungal agent. Secondary resistance is obtained byformerly sensitive fungal strains after they are exposed to the antifungal agent owing to altered gene expression. Another type of antifungal resistance is clinical resistance, which is failure of therapy or reoccuring of an infection by a fully susceptible fungal isolateto the antifungal agent used [15,16].

Specific mechanisms leading to antifungal resistance are enhanced efflux of drug by up-regulation of multidrug transporter genes resulting in reduced drug concentrations (e.g. azoles, allylamines); target site alteration by occurrence of mutations resulting in lower binding capacity of drug to target site (e.g. azoles); up-regulation of target enzyme through amplification of the respective gene, highertranscription rate, or lower degradation rate of the gene product (e.g. azoles); development of bypass pathways by alteration of specific steps in the ergosterol biosynthetic pathway (e.g. azoles and polyenes); decreased uptake of drug by decreasing permeability against drug (polyenes and pyrimidine analogues); decreased conversion to toxic antimetabolites (pyrimidine analogues). Table 1.3 shows possible drug resistance mechanisms in *T. rubrum* [13,15,17,18].

**Table 1.3** Probable mechanisms of drug resistance in *Trichophyton rubrum* [18].

| Drugs | Mechanism of action | Putative resistance mechanisms |
|---|---|---|
| Terbinafine | Inhibition of squalene epoxidase | Modification of target enzyme by mutation |
| | | Increased drug efflux |
| | | Stress adaptation[a] |
| | | Over-expression of salicylate mono-oxygenase (drug degradation) ? |
| Fluconazole | Inhibition of cytochrome P450 14α-lanosterol demethylase | Increased drug efflux |
| | | Stress adaptation[a] |
| Imazalil | Inhibition of cytochrome P450 14α-lanosterol demethylase | Increased drug efflux |
| Itraconazole | Inhibition of cytochrome P450 14α-lanosterol demethylase | Increased drug efflux |
| Ketoconazole | Inhibition of cytochrome P450 14α- lanosterol demethylase | Increased drug efflux |
| | | Over-expression of lanosterol-14α-demethylase |
| Tioconazole | Inhibition of cytochrome P450 14α- lanosterol demethylase | Increased drug efflux |
| | | Stress adaptation[a] |
| Amphotericin B | Binding to ergosterol and destabilization of cell membrane functions | Increased drug efflux |
| | | Stress adaptation[a] |
| Griseofulvin | Inhibition of mitosis | Increased drug efflux |
| | | Stress adaptation[a] |

[a] Stress adaptation: nonspecific responses to drug challenge

### 1.6 Side Effects of Commonly Used Antifungal Agents

Another problem associated with antifungal therapy is side effects of antifungal drugs (Figure 1.3). Especially, highlyimmune-compromised patients are treated with systemic antifungal agents for longer periods of time. Thus, there are longer-terms risks, including drug–drug interactions because of drug metabolism by the cytochrome P-450 system, organ dysfunction, and serious life-threatening skin diseases such as Stevens–Johnson syndrome, cutaneous reactions and malignancies as well as the dose-limiting toxicities.Commonly seen side effects of antifungal agents are gastrointestinal (GI) disturbances (nausea,abdominal pain, vomiting, and diarrhea) and hepatotoxicity,nephrotoxicityfever, chills, headache, rigors, respiratory problems, and hypertension, hypokalemia, hypomagnesemia, and metabolic acidosis related toelectrolyte abnormalities [14,19].

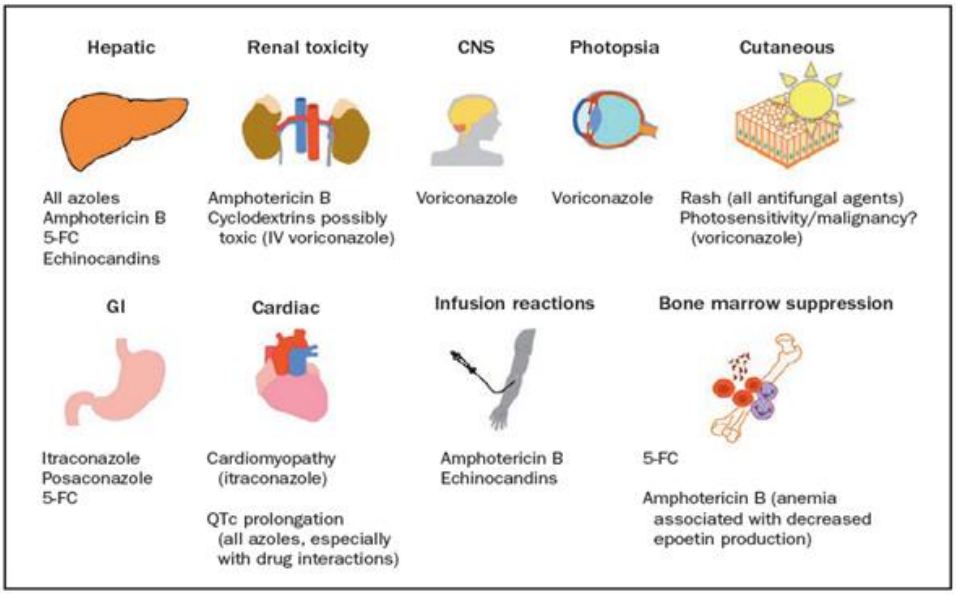



**Figure 1.3** Common toxicities of antifungal agents[14].

However, topical medications are considered as a safer therapy than systemic medications due to minimal serum absorption. Topical antifungal drugs are helpful for preventing further spread of infection and complete removal of infection. They are available without prescription and can be applied in various forms such aspowders, creams, liquids, aerosols sprays and ointments. There are only mild and transient adverse effects of topical antifungal drugs such as skin reactions at the application site which can be removed with termination of therapy [20].

To sum up, the limitations of current antifungal drugs are the absence of host selectivity and a higher toxicity profile, a narrow spectrum of activity, fungistatic effect instead of fungicidal activity, ineffectiveness against new or reemerging pathogenic fungi and the rapid development of drug resistance. Especially, antifungal agents that inhibit protein, RNA, or DNA biosynthesis have greater potential of toxicity for humans since fungi and human are both eukaryotic and they are very similar in respect of metabolic and signal transduction pathways. However, fungal cell wall seems to be a suitable target for antifungal agents because cell walls don't exist in mammalian cells [11,14].

### 1.7 Structure of Fungal Cell Wall

The fungal cell wall has unique components of the fungi, such as alpha and beta glucans, mannoproteins, and chitin (Figure 1.4). It is a complex structure containing mainly highly glycosylated glycoproteins (mannoproteins), two types of β-glucans, and chitin. For almost all fungi, the central core of the cell wall is a branched β-1,3-glucan and β-1,6-glucan that is linked to chitin via a β-1,4 linkage. There is also an extensive cross-linking between chitin, glucan and other wall components. The cell wall of fungi has important roles in such as protection as an initial barrier against hostile environments, cell morphology and rigidity, interaction with the host and resistance to immune functions mediated by host cell[21,22].

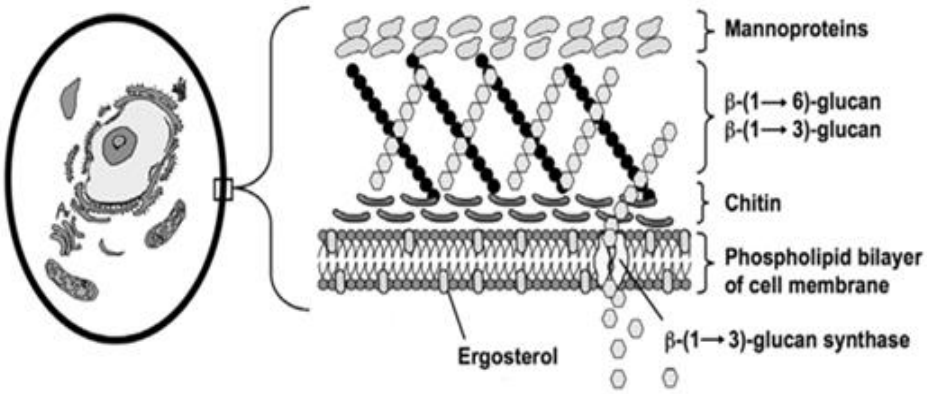



**Figure 1.4** Fungal cell wall components [19].

Because of the limitations of current antifungal agents, there is continuing need for the discovery of novel antifungal agentswith a unique mechanism of action. Antifungal agents targeted against fungal cell wall compounds have certain advantages over the modes of action of other antifungal agents in that they are toxic only to the fungi and the cross-resistance with other antifungal drugs does not occur. Their broad spectrum of fungicidal activity with trivial toxicity towards mammalian cells and ability to defeat the resistant strains that are now emerging as a result of therapy with existing antifungal drugs with a low tendency to evoke resistancemakes them a promising, attractive novel group of antifungals [12,23,24].

### 1.8 Yeast Killer Toxins

Specific yeast killer toxins are naturallyproduced and secreted by killer yeast strains and they are considered as potentialantifungal agents in the medical field. They are lethal to sensitive yeast cells and their related genera but not lethal to their own genera due to their self-immunity mechanisms. Yeast killer toxins are low molecular mass proteins or glycoproteins causing death of sensitive yeast and fungal strains without cell–cell contact that is an efficient way to compete for limiting resources in the environment. Studied yeast killer toxins are generally heat-labile, susceptible to proteases, and display their activity within narrow pH and temperature ranges, and showing their activity at acidic conditions[25-28].

Numerous yeast killer toxins have been described having different mode of action, molecular structure and maturation processes.Their common mechanisms of action includes hydrolyzing or inhibiting the synthesis of β-1,3-glucans which are the major cell wall componentsand forming ion channels on cytoplasmic membrane leading to ion leakage. Other mechanisms are blocking the DNA synthesis and budding cycle, and arrestingthe cell cycle in G1 phase [29].

### 1.9 Applications of Yeast Killer Proteins

During the last two decades, there have been many studies and suggestions about potential biotechnological applications of the killer yeasts and their toxins (Table 1.4). For instance, killer yeasts have been used as a starter culture to combat contaminating yeasts forthe production of wine, beer and bread, and they also have been used as biocontrol systems for the food preservation against spoilage yeasts. Moreover, yeast killer proteins have been suggested as novel and potent antimicrobial agents against human and animal fungal infections[26,27,29].

**Table 1.4** Potential applications of yeast killer toxins [26].

| Biotechnological field of application | Application |
|---|---|
| Biological control in agriculture | Antifungal activity against wood-decay and plant pathogenic fungi. Prevention of aerobic spoilage of silage. |
| Beverage fermentations | Avoid undesired contaminants in wine, beer, sake, etc. |
| Cellular biology research on eukaryotic cells | Studies of biosynthesis, cellular processing and secretion of proteins. |
| Food technology | Food preservatives of natural origin |
| Genetics | Selection of hybrids obtained by protoplast fusion; Fingerprinting of wine yeasts. Recombinant DNA technology (cloning vectors) |
| Medicine | Zymocide activities against pathogens |
| Taxonomy | Killer toxin sensitivity patterns may be indicative of phylogenetic affiliation. |

### 1.10 *Pichia anomala*, K5 Type Killer Yeast

Among killer yeast genera *Pichia spp*. represent valuble yeasts in terms of both fundamental and applied aspects. They are useful in studies of organelle biogenesis, structure and function and importantin production of human therapeutic proteins and biofuels, in food fermentations and in biocontrol agents. Especially, *Pichia anomala*, recently renamed as *Wickerhamomyces anomalus*,draws attention due to its variable characteristics. For example, in nature it has a wider range of habitats than well known *Saccharomyces cerevisiae*andshows a great diversity in its metabolism, morphology and stress-tolerance. Furthermore, *P. anomala* killer toxins exhibit broad spectrum antimicrobial activities against many fungi, yeast and bacteria and viruses (Table 1.5) [30,31].

**Table 1.5** Antimicrobial properties of *P. anomala* [31].

| Antimicrobial characteristic | Examples of microbes suppressed |
|---|---|
| Antifungal | *Aspergillus, Botrytis, Penicillium, Fusarium* |
| Antizymal | Various yeasts, incl. *C. albicans* |
| Antibacterial | *Erwinia* spp.; *Enterobacteriaceae*; *Streptococci* |
| Antiviral | Influenza virus |

In particular, killer toxin produced by *P. anomala* NCYC 434 has a wide range of killing activitywith a much higher growth inhibitory effect against fungiwith the cell walls containing a high proportion of β-1,3-glucan[29]. Some of the potentialbiotechnologcial applications of *P. anomala* are summarized in Table 1.6.

**Table 1.6** *P. anomala* products of biotechnological potential [31].

| Product | Potential application |
|---|---|
| Sophorolipids | Biosurfactants |
| γ-aminobutyric acid, GABA | Pharmaceuticals (GABA acts as a neurotransmitter, improves cerebral blood flow) |
| Volatile organic compounds | Fragrances |
| Isobutanol | Biofuels |
| Beverage starter culture | Low-alcohol wines; aromas |
| Panomycocin | Novel zymocidial agents |
| Antiviral agent | Influenza virus therapy |
| Amoebicidal agent | Therapy of *Acanthamoeba* infections |
| Anti-*Pneumocystis* agent | Therapy of *Pneumocystis carnii* |
| Antibacterial agent | Therapy of Streptococcal infections |
| Biocontrol/ biopreservative | Stored grain, vines, fruit |
| Enzymes | Phytase, esterase, peptidase, β-glucosidase, amylase |
| Bioethanol (indirectly) | Maintenance of airtight stored grain (biofuels) |

### 1.11 K5 Type Yeast Killer Protein, Panomycocin

Recently, the killer protein of *P. anomala* NCYC 434 has been purified, characterised and named as "Panomycocin". Itis a 49 kDamonomeric glycoprotein with high stability at pH 3-5.5 and temperatures up to 37 °C. It isan enzyme with exo-β-1,3-glucanase activityso its mechanism of killing is to hydrolyse the β-1,3-glucans which are essential polymers for fungal cell wall tomaintain its integrity. As a result of hydrolysis of this polymer, fungal cell wall is destroyed leading to death of the sensitive cells [32,33].

*In vitro* activity of Panomycocin was examined against nine strains of dermatophytes, *Microsporum audouinii, M. canis, M. gypseum, Trichophyton equinum, T. interdigitale, T. mentagrophytes, T. rubrum, T. tonsurans, T. verrucosum*and all of the strainswas found to be susceptible to Panomycocin. Since Panomycocin is highly active *in vitro* against fungal strains that cause superficial infections, it has been proposed a potent topical antifungal agent with novel mechanism of action [34]. Moreover, in other studies Panomycocin was found to have the *in vitro* killingactivity against human isolates of pathogenic *Candida spp.*causing candidiasis [35] and *in vitro* and *in vivo* activitiesagainst isolates of*Penicillium digitatum* and *Penicillium italicum*causing green mold and blue mold diseases in citrus fruits [36].

Nowadays, therapeutic proteinshave gained increasing importance as new therapeutic agents in the treatment of superficial dermatophytosisdue to their highly specific mechanisms of action, relatively lower dose requirements and their high efficacy. Thus, Panomycocin has a high potential as a therapeutic antifungal protein for the treatment of superficial dermatophytosis owing to its novel mechanism of action, high selectivity and high stability at skin conditions.

### 1.12 Protein Delivery Systems

Protein drugs differfrom traditional ones in terms of molecular size, biological and physicochemical stability, bioavailability and dose requirement. The effective formulation of proteins appears as an important challenge due to their delicate structure which relies on non-covalent interactions such as electrostatic and hydrophobic interactions, hydrogen bonding and van der Waals forces. Physical (denaturation, aggregation, precipitation) or chemical factors (oxidation, hydrolysis, deamidation, disulfide exchange)may causedestabilization the proteins. Therefore, harmful manufacturing procedures and enxternal factors such as sterilization, lyophilisation, unfavorable pH, ionic strength and temperature, high pressure,shear force and agitation, detergents, non-aqueous solvents and metal ions .may disturb the chemical and physical stability of proteins, impair biological activity, make them immunogenic and cause their aggregation and precipitation.Moreover, since proteins are highly biodegradable by enzymes and proteases which can be found at the administration site and inside the body, they have a short *in vivo* half life making them poorly bioavailable.For the mentioned reasons, it is important to preserve protein stability, prolong their half life and increase their absorption. To overcome these limitations and improve pharmacological properties of protein drugs,applications of proteins with drug delivery systems are gaining increasing importance. Main advantages of protein delivery systems are; (1) enhanced protein solubility, stability, bioactivity and bioavailability by coating and isolating; (2) controlled release of protein at a predetermined rate; (3) improved biodistribution of protein; (4) target the diseased tissue *in vivo*; (5) improved patient convenience; (6) improved efficiency with reduced toxicity; and (7) administration of lower dose of proteins due to decreased drug clearance [37-40].

### 1.13 Routes of Protein Drug Delivery

The routes of protein drug administration can be classified as parenteral and nonparenteral. For the systemic delivery of therapeutic proteins, parenteral administration is currently believed to be efficient delivery route to achieve therapeutic activity. Parenteral delivery consist of three major routes: intravenous(IV), intramuscular(IM), subcutaneous(SC). Among them intravenous administration, direct injection into the blood, is currently the method of choice for systemic delivery of proteins. The advantages of parenteral route are the rapid achievement of concentration in the blood and precision in delivering the effective dosage. Among the disadvantages, are the high initial concentration that can fall into toxic levels, especially for drugs with a low therapeutic index, the fact that once the drug is injected there is no retreat and the short halftime of the protein, which results in the necessity of frequent administrations to obtain therapeutic effectiveness. Due to these disadvantages, nonparenteral protein delivery systems are extensively investigated. Nonparenteral systemic routes include oral, nasal, buccal, ocular, rectal, transdermal, pulmonary, and topical routes; however, problems associated with these routes include low systemic bioavailability of drugs, ability to pass across intestinal cell membranes between the lumen of the gastrointestinal (GI) tract and the body fluids, and endurance to destruction by hepatic metabolism (first-pass effect). For these reasons topical route is preffered for dermatophytosis and use of topical agents increased in recent years due to various advantages over systemic treatment which are no first pass metabolism, little or no risk of systemic side-effects or drug interactions, specific targeting to the site of the lesions, drug efficacy with lower dosage of drug, no fluctuation in drug levels, and convenience to use than oral medications with more patient compliance [37,41-44].

### 1.14 Chitosanas aPolymeric Protein Delivery System

For the last two decades, polymeric nanoparticles are gaining importance as drug carriersystems to their advantages such as their ability to protect and deliver drugs or macromolecules and release in a controlled fashion.However, for biocompatibility and biodegradability reasons, a very limited numbers of synthetic or natural polymers can be used for pharmaceutical applications. Recently, natural chitosan polymer has received great prominence in biomedical and pharmaceutical areasowing to its useful physicochemical and biological properties. It has been recognized as a promising natural polymer for especially delivery of sensitive macromolecules like therapeutic proteins and genes [45,46].

#### 1.14.1 Physicochemical Properties of Chitosan

Chitosan is a natural linear biopolyaminosaccharide having both glucosamine (β-(1→4)-linked 2-amino-2-deoxy-D-glucose) and N-acetylglucosamine (2-acetamido-2-deoxy-D-glucose) units. It is obtained by partial alkaline deacetylation of chitin (composed of only β-(1→4)-linked *N*-acetyl-glucosamine units) (Figure 1.5), which is the second abundant polysaccharide after cellulose and found in protective cuticles of crustaceans such as crabs, shrimps, prawns and lobsters [47,48].

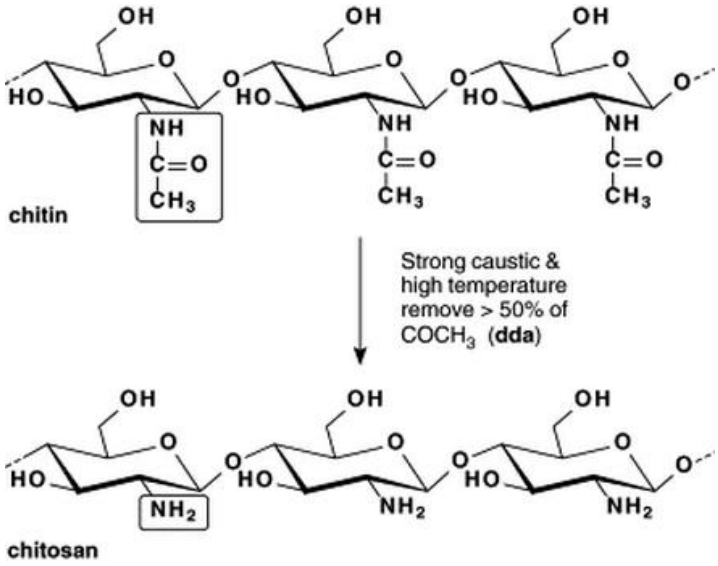



**Figure 1.5** Synthesis of chitosan from chitin [49].

There are different chitosan polymers available in terms of molecular weights (50– 2000 kDa), degree of deacetylation (40–98%) and viscosity ranging between 20-2000mPas. Chitosan appears as colorless, odorless flakes.Chitosan has a high positive charge density so it can adhere to negatively charged surfaces and chelate metal ions. It also acts as a viscosity enhancing material in an acidic environment dueto its high molecular weight and a linear unbranched structure[50,51].Physicochemical properties of chitosan are summarized in Table 1.7.

**Table 1.7** Summary of physicochemical properties of chitosan [51].

| **Properties** | | |
|---|---|---|
| Physical | Particle size | $< 30\ \mu$m |
| | Density | 1.35–1.40 g/cc |
| | pH | 6.5–7.5 |
| | Solubility | Insoluble in water but soluble in acids |
| Chemical | Cationic polyamine | |
| | ● High charge density at pH < 6.5 | |
| | ● Adheres to negatively charged surfaces | |
| | ● Forms gels with polyanions | |
| | High molecular weight, linear polyelectrolyte | |
| | ● Viscosity – high to low | |
| | Chelates certain transitional metals | |
| | Amiable to chemical modifications | |
| | ● Reactive hydroxyl/amino groups | |

Chitosan can be modified chemically due to its reactive groups which are primary and secondary hydroxyl groups on each unit and the amino group on each deacetylated unit.Also, mechanical and physical properties of chitosan can be modified by chemical modifications. Chitosan is a weak base (pKa 6.2–7) and not soluble in water or in organic solvents. It can be dissolved in dilute aqueous acidic solution (pH <6.5) in which amine groups of chitosan become protonated, ($R\text{-}NH3^{+}$) (Fig1.6). Thus,positive charge of chitosan polymer enables itto interact with negatively charged surfaces in aqueous environments [48,52-54].

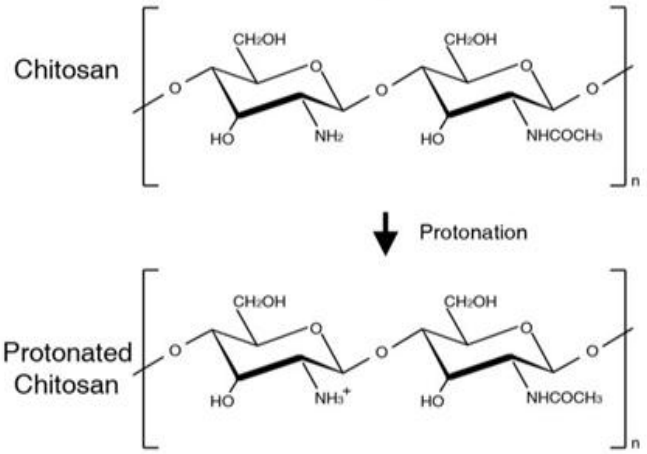



**Figure 1.6** Molecular structures of chitosan and protonated chitosan polymer [55].

### 1.14.2 Biological Properties of Chitosan

Chitosan has many favorable biological properties which are biocompatibility, biodegradability, nontoxicity [54-58] along with other reported properties such as analgesic [59], immunity-enhancing, antitumor, and anticancer effects [58-60], antiviral [61], antibacterial [62], hypocholesterolemic [63], hemostatic [64], wound-healing [65-67] and antioxidant [68].

Chitosan can be degraded *in vivo* by proteases such as lysozyme and pepsininto non-toxic productswhich can be incorporated to glycosaminoglycans and glycoproteins or to metabolic pathways. It is considered as safe since it has the LD50 (lethal dose 50%) very similar to the salt and glucose values in assays carried out on mice and also is proven to be safe in rats up 10% in the diet [54].

Chitosan has been used inmucoadhesive formulationsto enhance drug absorption through mucoadhesion, due to its positive charge that interacts with mucus and negatively charged sialic acid residues on the mucosal surface. Also it has gelling ability in aqueous environment which is favorable for interpenetration of polymer and glycoprotein chains into mucous.

Another important property of chitosan is its ability to increase epithelial permeability of drugs both *in vivo* and *in vitro*and facilitate the transport of macromolecules through epitheliaby the rearrangement of tight junction proteins and opening the intercellular tight junctions transiently. Several mechanisms suggested for the permeation enhancing behaviour are the electrostatic interaction between its protonated amine groups andnegatively charged groups of cell membrane leading to a reversible structural reorganization of the tight junction-associated proteins, interaction of chitosan with the Protein Kinase C pathway, redistribution of cytoskeletal F-actin and translocation of the tight junction proteins, ZO-1 and occluding, from the plasma membrane. Thus, chitosan improves the drug bioavailability due to its absorption enhancing effect and facilitates the drug uptake through the cell membrane [48,54,56,69,70].

### 1.15 Chitosan Nanoparticles

Chitosan has been extensively studied for its potential in the development of drug delivery systems in various forms depending on the drug and its destination such as tablets gels, films, emulsions, capsules, beads, hydrogels, scaffolds, membranes, sponges, microspheres, nanoparticles and microcapsules (Table 1.8) [71-73].

**Table 1.8** Chitosan-based drug delivery systems, their methods of preparation for various drugs [71].

| Type of system | Method of preparation | Drug |
|---|---|---|
| Tablets | matrix | diclofenac sodium, pentoxyphylline, salicylic acid, theophylline |
| | coating | propranolol HCl |
| Capsules | capsule shell | insulin, 5-amino salicylic acid |
| Microspheres/Microparticles | emulsion cross-linking | theophylline, cisplatin, pentazocine, phenobarbitone, theophylline, insulin, 5-fluorouracil, diclofenac sodium, griseofulvin, aspirin, diphtheria toxoid, pamidronate, suberoylbisphosphonate, mitoxantrone, progesterone |
| | coacervation/precipitation | prednisolone, interleukin-2, propranolol-HCl |
| | spray-drying | cimetidine, famotidine, nizatidine, vitamin D-2, diclofenac sodium, ketoprofen, metoclopramide-HCl, bovine serum albumin, ampicillin, cetylpyridinium chloride, oxytetracycline, betamethasone |
| | ionic gelation | felodipine |
| | sieving method | clozapine |
| Nanoparticles | emulsion-droplet coalescence | gadopentetic acid |
| | coacervation/precipitation | DNA, doxorubicin |
| | ionic gelation | insulin, ricin, bovine serum albumin, cyclosporin A |
| | reverse micellar method | doxorubicin |
| Beads | coacervation/precipitation | adriamycin, nifedipine, bovine serum albumin, salbutamol sulfate, lidocaine–HCl, riboflavin |
| Films | solution casting | isosorbide dinitrate, chlorhexidine gluconate, trypsin, granulocyte-macrophage colony-stimulating factor, acyclovir, riboflavine, testosterone, progesterone, beta-oestradiol |
| Gel | cross-linking | chlorpheniramine maleate, aspirin, theophylline, caffeine, lidocaine–HCl, hydrocortisone acetate, 5-fluorouracil |

Among these forms, chitosan nanoparticlespresentsmany advantages;

- better formulation stability and reproducibility,
- use of mild preparation methods,
- avoidance the use of harmful organic solvents,
- readily available for cross linking,
- ability to lyophilize,
- protection of proteins from enzymatic degradation,
- controling the release of active agents,
- exhibiting absorption enhancing effect,
- enhancing the interaction of proteins with epithelial cell membranes and/or mucus,
- prolonging the duration of therapeutic effect at thetarget site.

### 1.16 Methods of Preparation of Chitosan Nanoparticles

Several techniques have been used to prepare chitosan NPs and the most common techniques are ionotropic gelation, microemulsion, chemical cross-linking, the emulsification solvent diffusion, polyelectrolyte complex. Among these techniques, chitosan nanoparticles prepared with ionotropic gelation isa very promising system and received much attention in recent years for the encapsulation and delivery of sensitive macromolecules, especially for proteins such as bovine serum albumin (BSA), insulin, tetanus toxoid, diphtheria toxoid. Ionotropic gelation method is simple procedure and also mild to proteins which involvesreversible physical cross-linking instead of using chemical crosslinkers such as use of formaldehyde and glutaraldehyde which are toxic and also avoids the use of organic solvents, high shear force and high temperatures. The protein and chitosanare mostly in associated via electrostatic and hydrophobicinteraction, and hydrogen bonding [74-78].

#### 1.16.1 Ionotropic Gelation Method

Chitosan NPs preparation by ionotropic gelation metod was first reported by Calvo *et al.*, (1997)and then this method has been studied and developed in various aspects [79-88]. Ionotropic gelation method uses the ability of chitosan totransit from liquid to gel phase upon contact with special polyanions by inter and intra cross-linkages between/within polymer chains due to electrostatic interaction between amine groups of chitosan and negatively charge groups of a polyanion such as tripolyphosphate in the aqueous environment. This simple and mild method involves the dissolving of chitosan in acetic acid and then addition of polyanion or anionic polymers resulting in spontaneous formation of nanoparticles of small size in the range of 200-500 nm under mechanical stirring at room temperature [79-89].

Especially for preparationof protein and antigen-loaded chitosan NPs, the most commonly used counterion for ionotropic gelation is sodium tripolyphosphate, TPP (Figure 1.7), which is a small ion with a triple negative charge when dissolved in water. Moreover, it was shown that the crosslinking by TPP makes the nanoparticles more condense and more resistant to the freeze-drying [48,76,77].

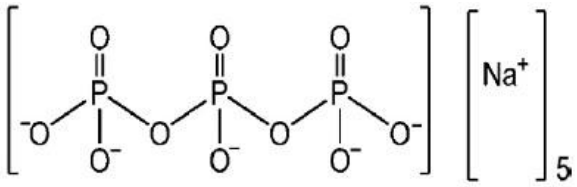



**Figure 1.7** Molecular structure of sodium tripolyphosphate[90].

By ionotropic gelation technique with TPP, nanoparticles are formed byinter and intra molecular linkages between negatively charged phosphate groups of TPP and protonated amine groups of chitosanupon mixing ofan alkaline phase (pH 7–9) of TPP with an acidic phase (pH 4–6) of chitosan (Figure 1.8).For a high yield of nanoparticles, the concentration of both chitosan and TPP should be controlled at a suitable range and generally chitosan:TPP weight ratio was found to be within the range of 3:1–6:1, commonly 5:1 and a volumetric ratio of 2.5 : 1 (v/v) (chitosan:TPP) have been used [75,76,91].

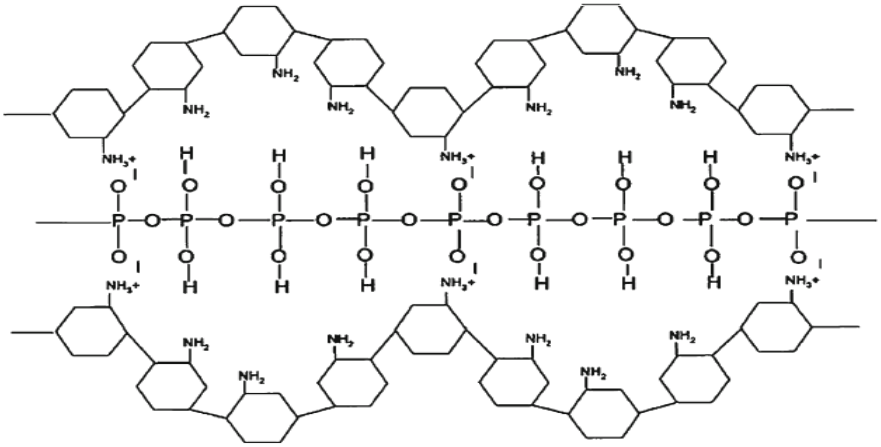


**Figure 1.8** Ionotropic cross-linking of chitosan and TPP [92].

By changing parameters such as the concentration or molecular weight of chitosan, the pH of the reaction medium, the cross-linking time and the ratio of the chitosan to the crosslinker, nanoparticle size and surface morphology, surface charge of particles,protein encapsulation efficiency and release profiles can be modified [45,74].

### 1.17 Protein Drug Release

*In vitro* release of drug depends largely upon volume of release medium, pH and polarity of dissolution medium, rate of stirring, temperature, sink condition and presence of enzyme.There are three main mechanisms for protein release from chitosan nanoparticles;(a) desorption of protein molecules, (b) diffusion of protein from nanoparticlesand (c) release upon erosion of polymer.

Most of the time, these mechanisms occurs either simultaneously or in sequence. Desorption of protein from the nanoparticle surface involves dissolving of the adsorbed drug when it contacts with the release medium which leads to burst effect. Drug release by protein diffusion from nanoparticles involves penetration of water into particulate system causing swelling of the matrix and the diffusion of drug from the swollen matrix. To sum up, chitosan nanoparticles formed by ionotropic crosslinking with TPP for the delivery of Panomycocin has some interesting features, mainly: (i) nanoparticle formation under mild conditions for protection of protein stability; (ii) a positive nanoparticle surface charge that can attract negatively charged protein; (iii) a great intrinsic capacity to enhance formulation efficiency; (iv) providing controlled release of the active protein at skin; (v) increasing retention time and concentration of protein at skin due to biadhesive property of nanoparticles; and finally (vi) retention of their integrity of nanoparticles and the activity of the protein upon freeze-drying and reconstitution [93-95].

### 1.18 Aim of the study

The aim of the study is to achieve novel therapeutic topical formulation of potential antifungal agent K5 killer toxin, Panomycocin, with CS-TPP nanoparticles to treat dermatophytosis (tinea infections). Panomycocin loaded CS-TPP nanoparticles were prepared by ionotropic gelation method and characterizations of these NPs were done in terms of surface morphology and particle size, zeta potential, molecular structure, association efficieny and loading capacity. Moreover, in this study, *in vitro* release of Panomycocin from chitosan-TPP nanoparticles was investigated and *in vitro*antifungal activity of the Panomycocin-loaded chitosan-TPP nanoparticles against common human dermatophytes was determined.

# CHAPTER 2

# MATERIALS AND METHODS

## 2. 1 Materials

### 2.1.1 Fungal Strains

The source of the K5 type yeast killer protein, *Pichia anomala*, (NCYC 434, K5) and sensitive strain, *Saccharomyces cerevisiae* (NCYC 1006) were purchased from the National Collection of Yeast Cultures, Norwich, U.K. Pathogenic fungal strains which were used in antifungal susceptibility studies are given in Table 2.1.

**Table 2.1** Dermatophyte strains used in antifungal susceptibility studies.

| **Strain** | **Strain number\Source** | **Isolation** |
|---|---|---|
| *M. gypseum* | DSMZ 3824 | Human |
| *Trichophyton rubrum* | DSMZ 4167 | Human |
| *T. mentagrophytes* | DSMZ 4870 | Tinea pedis |
| *T. interdigitale* | DSMZ 12283 | Tinea pedis |
| *T. equinum* | DSMZ 12284 | Human skin |
| *T. tonsurans* | DSMZ 12285 | Human skin |

DSMZ: German National Resource Centre for Biological Material

### 2.1.2 Culture Media

For the maintenance and routine growth, fungal cells were grown in YEPD medium (pH 5.5) consisting of 1 % Bacto-yeast extract, 1 % Bacto-peptone and 2 % dextrose along with 2 % Bactoagar or Sabouraud medium (pH 5.6) containing 1 % Bacto-peptone and 2 % dextrose along with 2 % Bacto-agar.

For the production of Panomycocin, *P. anomala* cells were grown in YEPD medium buffered to pH 4.5 with phosphate citrate buffer with the addition of 5 % glycerol. Killer activity was determined in YEPD medium with 2 % Bacto-agar buffered to pH 4.5 with phosphate-citrate buffer.

Prior to antifungal susceptibility studies all pathogenic fungal cells were subcultured in potato dextrose agar (0,4 % potato extract, 2 % dextrose and 5 % agar) plates to ensure purity and viability.

### 2.1.3 Nanoparticle Preparation

Medium molecular weight chitosan, (MWM, 190kDa-310kDa) with degree of deacetylation of 75-85% and sodium tripolyphosphate (TPP) were purchased from Sigma–Aldrich, (USA).

### 2.1.4 Chemicals

The chemicals and the suppliers are listed in the Appendix A.

### 2.1.5 Buffers

Buffers and solutions used in the experiments are given in Appendix B.

### 2.1.6 Instrumentation

Gel filtration chromatography was done by using FPLC system (Biocad 700E Perseptive Biosystems, USA) including an automatic fraction collector (SF-2120 Super Fraction Collector, Advantec MFS, Japan) with TSK G2000 SW (7, 5 mmD/300mmL TosoHaas, Japan) column.

Mechanical stirrer Heidolph MR 3001, Heidolph Instruments GmbH & Co. KG, Germany was used for preparation of NPs.

Amount of released drugs were detected spectrophotometrically by using Nanodrop 2000 UV-Vis Spectrophotometer, Thermo Fisher Scientific, USA.

Loaded and unloaded chitosan NPs were freeze dried by using a freeze drier, Christ Alpha 1-4 LD plus, Germany.

Particle size and surface morphology of NPs were examined by Scanning Electron Microscope, QUANTA 400F Field Emission SEM, USA.

Zeta potential of NPs was measured by Zetasizer, MALVERN Nano ZS 90, UK.

NPs were examined with Fourier Transform Infrared Spectroscopy, FTIR,Bruker Elexsys E580, Germany.

## 2.2 Methods

### 2.2.1 Sterilizations

The glassware, the media for stock cultures and for routine growth of the yeast cells, buffers and distilled water used in all steps were sterilized at 121 $^{o}$C for 15 minutes on liquid cycle. Chitosan solution was filtered through 0.45 μm syringe filter with cellulose acetate membrane. TPP and lactose solutions were filtered through 0.22 μm cellulose acetate syringe filter (Sartorius, AG, Germany). RPMI 1640 medium was filtered through 0.45μm and 0.22μm (Sartorius, AG, Germany) cellulose acetate filters respectively for sterilization.

### 2.2.2 Maintenance of the Fungal Cultures

Master stock cultures of *Pichia anomala* (NCYC 434) and *Saccharomyces cerevisiae* (NCYC 1006) were maintained in YEPD agar plates were stored at +4 $^{o}$C and used to propagate new master stocks and to replace the working stock in every 2 months [96].

Active cultures of dermatophyte strains in glass tubes were covered with adequate amount of sterile saline solution. Spores were scratched with the tip of a sterile pasteur pipette and 0.5 ml of the spore suspension was drawn under sterile conditions and plated onto YEPD pH 5.5 or SDA pH 6.0 agar plates and incubated at 28º C. Dermatophyte strains were sub-cultivated to PDA plates to promote spore formation prior to antifungal susceptibility testing.

### 2.2.3 Production of the K5 Type Yeast Killer Toxin

Production, concentration and isolation of the K5 type yeast killer protein were done as described previously by İzgü and Altınbay [32]. *Pichia anomala* NCYC 434 cells were cultivated into 10 ml of YEPD pH 5.5 medium and incubated overnight at 25 $^{o}$C. One ml of cell suspension was further inoculated into 100 ml of the same medium. After the yeast cells were incubated at 25 $^{o}$C overnight at 120 rpm on a gyratory shaker (Innova 4330, New Brunswick Scientific, USA), 10 ml of cell suspension was transferred to 1L of YEPD medium (containing 5 % glycerol) adjusted to pH 4.5 with acetic acid and incubated until stationary phase at 20 $^{o}$C for 48 hours at 120 rpm on a gyratory shaker (Innova 4330, New Brunswick, USA). Centrifugation (KR 22i, Jouan, France) at 9000 rpm for 15 min. at 4 $^{o}$C was applied to obtain the cell free culture medium and supernatant was filtered through 0.45μm and 0.2μm cellulose acetate membranes (Sartorius, AG, Germany) respectively for the sterilization of the medium.

### 2.2.4 Preparation of Crude K5 type Killer Toxin

Cell free culture medium containing the K5 type yeast killer protein was concentrated 50 fold by using first 30 kDa and then 5 kDa molecular weight cut-off ultrafiltration systems (Vivaflow 200, Sartorius AG, Goettingen, Germany) having polyethersulfone (PES) membranes operating with peristaltic pump at pressure approximately 2.5 bar.

### 2.2.5 Determination of Killer Toxin Activity

At various stages of the study killer toxin activity was tested according to İzgü and Altınbay [32] with an agar diffusion assay. Twenty five ml of molten YEPD agar (pH 4.5) was seeded with 0.5 ml of *Saccharomyces cerevisiae* (NCYC 1006) cells in sterile water at a cell density of a 0.5 McFarland standard($1\times10^6$ to $5\times10^6$ cells/ml) and poured into petri dishes. Protein samples of 50 µl were spotted onto petri dishes and incubated at 25$^{o}$C. The killer activity was determined by visualizing the clear zone of growth inhibition after 48 hours of incubation.

### 2.2.6 K5 type Killer Toxin Purification by Gel Filtration Chromatography

Concentrated crude toxin was subjected to gel filtration chromatographies by using a fully automated FPLC system (Biocad 700E Pseerptive Biosystems, USA) including an automatic fraction collector (SF-2120 Super Fraction Collector, Advantec MFS, Japan). Detections were done with UV absorbance at 280 nm at 20 $^{o}$C.

The concentrated and buffer exchanged protein sample was subjected to gel filtration chromatography using a TSK G2000 SW (7, 5 mmD/300mmL TosoHaas, Japan) column. Prior to injection of the sample, column was equilibrated with 100mM $Na_2HPO_4$- citric acid buffer, pH 4.5, containing 100mM $Na_2SO_4$ at a flow rate of 1 ml/min. 90 µl of sample was injected into the column and elution was done with the same buffer at a flow rate of 1 ml/min. Killer toxin containing eluted fractions (1,3 ml) that corresponds to 8.5 ml were pooled.These active fractions are then concentrated and buffer exchanged with acetate buffer of pH 4.5 by using 5 kDa molecular weight cut-off ultrafiltration system (Vivaflow 200, Sartorius, AG, Germany) having polyethersulfone (PES) membranes operating with peristaltic pump at pressure approximately 2.5 bar. Fifty µl of the purified protein obtained from gel filtration chromatography was spotted on to YEPD (pH 4.5) agar plates seeded with killer toxin sensitive *S. cerevisiae* NCYC 1006 cells for the assessment of the killer toxin activity.

### 2.2.7 Assessment of Protein Concentration

Protein concentration was determined as described previously by Bradford [97] with some minor modifications. Bradford reagent used for the determination of protein concentration was prepared as follows. A hundered mg of Coomassie Brilliant Blue G-250 was dissolved in 50 ml of 95 % ethanol then mixed with 100 ml of 85 % (w/v) phosphoric acid. Finally, the solution was completed to a total volume of 1 L with distilled water and filtered through Whatman #1 paper.

Bovine serum albumin (Fraction V) was used as the protein standard. Different concentrations of bovine serum albumin was prepared in 100 mM $Na_2HPO_4$-citric acid buffer ranging from 25 to 125 μg/ml in a total volume of 800μl. These standard solutions were mixed with 200μl Bradford reagent. 80μl protein sample was also diluted in 720μl 100 mM $Na_2HPO_4$-citric acid buffer and mixed with 200μl Bradford reagent. Absorbances were immediately measured at 590nm by using UV-visible spectrophotometer (model 1208, Shimadzu, Japan). Protein concentration was calculated using a standard curve of absorbance versus protein amount.

Also, protein concentration of killer toxin was determined by using UV-Vis spectrophotometer (Nanodrop 2000, Thermo Scientific, USA) at 280 nm.

### 2.2.8 SDS Polyacrylamide Gel Electrophoresis

Gel electrophoresis was performed by using SE250/SE260 Mighty Small slab gel unit (Hoefer, USA). Concentrated protein was electrophoresed on a 12.5 % linear, 0.75 mm thick polyacrylamide gel in a discontinuous buffer system using a vertical slab gel electrophoresis unit SE 250 (Hoefer, USA) to ensure the purity of the toxin using standard Laemmli [98] protocol.

12.5 % separating gel (Table 2.2) was prepared and poured into the electrophoresis unit and covered with water saturated n-butanol to avoid contact of the gel with air and left for polymerization for 1 hour. When the polymerization was completed n-butanol was washed with water and stacking buffer respectively before the stacking gel (Table 2.3) was poured. Stacking gel was also left for polymerization for 1 hour. The seperating and stacking gels were prepared according to Table 2.3 and 2.4.

Protein samples were subjected to acetone precipitation and resuspended in 125 mM Tris-Cl pH 6.8. Samples were heated at 100 °C for 5 min in equal volume of sample buffer (0.125 M Tris-Cl, 20 % (v/v) glycerol, 4 % (v/w) SDS, 0.02 % (v/w) bromophenol blue, pH 6.8) and 10 % 2-β- mercaptoethanol was added to the sample buffer for SDS-PAGE. The samples were loaded onto the gel after the polymerization of the stacking gel was completed.

Molecular mass markers include $\alpha_2$-2-macroglobulin (170,000 Da), β-galactosidase (116,353 Da), fructose-6-phosphate kinase (85,204 Da), glutamate dehydrogenase (55,562 Da), aldolase (39,212 Da), triose phosphate isomerase (26,626 Da), trypsin-inhibitor (20,100 Da), lysozyme (14,307 Da).

Electrophoresis was done at 15 mA/0.75 mm gel (Power supply PP4000, Biometra, Germany) at constant current for 1 h.

**Table 2.2** Seperating gel mixture.

| Components | 12.5% Gel |
|---|---|
| Acrylamide-bisacrylamide (30:0.8) | 8.3 ml |
| 4X Seperating Gel Buffer (1.5M Tris-Cl ,pH:8.8) | 5 ml |
| 10 % SDS | 0.2 ml |
| ddH2O | 6.4 ml |
| 10 % Ammonium persulfate* | 100 µl |
| TEMED* | 6.7 µl |

*APS and TEMED were added after deaeration.

**Table 2.3** Stacking gel mixture.

| Acrylamide-bisacrylamide (30.8%T 2.7%$C_{bis}$) | 0.44 ml |
|---|---|
| 4X Stacking Gel Buffer (0.5M Tris-Cl, pH:6.8) | 0.83 ml |
| 10 % SDS | 33 µl |
| ddH2O | 2.03 ml |
| 10 % Ammonium persulfate* | 16.7 µl |
| TEMED* | 1.7 µl |

*APS and TEMED were added after deaeration.

### 2.2.9 Protein Detection in Gels by Coomassie Brilliant Blue Staining

Protein bands on the gel were visualized by coomassie brilliant blue standard staining method described by Wilson [99]. After the gel was removed, it was placed in staining solution and incubated on rotary shaker overnight. After staining, gel was placed in destaining solution I to remove the bulk of the excess stain. Then gel was placed in destaining solution II and refreshed until the gel background was clear. The proteins were detected as blue bands on a clear background.

### 2.2.10Preparation of Blank and Panomycocin-loaded Chitosan-TPP Nanoparticles

CS-TPP NPs were prepared according to the procedure previously developed by Calvo et al. [100] with some modifications.MWM chitosan flakes were dissolved in 1% (v/v) acetic acid solution under magnetic stirring to make up chitosan concentration at 2 mg/ml (40 mg chitosan). Then chitosan solution was filtered through 0.45 µm cellulose acetate membrane to remove bulk particles. Then pH of chitosan solution was raised to 4.5 with 10N NaOH solution. TPP was dissolved in sterile distilled water to obtain TPP solution of 1 mg/ml (8 mg TPP) and was filtered through 0.2 µm cellulose acetate membrane. TPP solution, used

as a cross-linking agent, was added dropwise with a syringe to chitosan solution under magnetic stirring at room temperature. CS–TPP NPs were formed spontaneously upon addition of TPP solution into chitosan solution via the TPP initiated ionic crosslink/gelation mechanism. The NPs were formed at a selected chitosan to TPP weight ratio of 5:1 and volumetric ratio of 2.5:1 (v/v). The nanoparticle suspension was gently stirred for 50 min at room temperature. NPs were purified by centrifugation at 25000 xg for 45 min at 4 °C (Sigma 3K30, SciQuip Ltd, UK) and washed once with distilled water.

For encapsulation of Panomycocin, 1 ml of protein solution was slowly added to chitosan solution (pH 4.5) and stirred gently for 5 min then TPP solution was added dropwise. The nanoparticle suspension was gently stirred for 50 min at room temperature. NPs were purified by centrifugation at 25000 xg for 45 min at 4 °C (Sigma 3K30, SciQuip Ltd, UK) and washed once with distilled water.

After pellet obtained and resuspended in 1 ml of 5% (w/v) lactose solution for protection of NPs, samples were frozen overnight at −80°C. Then samples were freeze dried at −60 $^{o}$C for 48 h using freeze drier (Christ Alpha 1-4 LD plus, Germany). Both CS-TPP NPs and supernatant were stored at 4 $^{o}$C until further analysis.

### 2.2.11 Evaluation of Association Efficieny and Loading Capacity

After centrifugation of protein loaded CS-TPP nanoparticles, supernatant was taken and protein concentration in supernatant was determined with UV-Vis spectrophotometer (Nanodrop 2000, Thermo Scientific, USA) with absorbance at 280 nm. Then amount of protein associated with nanoparticles was determined by substracting the protein amount in supernatant from initial amount of protein. After that, protein association efficiency (A.E) and loading capacity (L.C) of nanoparticles were calculated according to the equations given below [84,101,102].

$$\% \text{ AE} = 100 \times \frac{\text{Total protein amount} - \text{Free protein amount}}{\text{Total protein amount}}$$

$$\% \text{ LC} = 100 \times \frac{\text{Total protein amount} - \text{Free protein amount}}{\text{Weight of nanoparticles}}$$

### 2.2.12 Surface Morphology and Particle Size of Blank CS-TPP NPs and Panomycocin-loaded CS-TPP NPs

The surface morphology and particles size of the freeze dried chitosan and CS–TPP NPs (with and without protein) were examined via scanning electron microscope (QUANTA 400F Field Emission SEM, USA). The powders were previously mounted on a brass stub using double-sided adhesive tape and made electrically conductive by coating with gold-palladium film in a vacuum. The chitosan and nanoparticles werescanned in a high vacuum chamber with a focused electron beam an excitation voltage of 5 kV at different magnifications (10000 to 100000x).

The particle size was estimated as the Feret's diameter (distance between two tangents on opposite sides of the particles) and was directly determined by analizing SEM, and was estimated as the mean of 100 particles measurement ($n$ = 100) [92,101-104].

### 2.2.13 Fourier Transform Infra Red Spectroscopy (FTIR)

The structure of CS and CS-TPP NPs (with and without protein) were evaluated by Fourier transform–infrared spectroscopy (FTIR, Bruker, Germany) at a resolution of 4 $cm^{-1}$and 16 scansin the range 4000–400 $cm^{-1}$ using KBr pellets [105,106].

### 2.2.14 Measurement of Zeta Potential of Blank CS-TPP NPs and Panomycocin-loaded CS-TPP NPs

The zeta potential values were calculated from the mean electrophoretic mobility of the particles were determined by laser-doppler anemometry (LDA)using Zetasizer® Nano ZS 90 (Malvern Instruments, UK). For determining the electrophoretic mobility, samples were placed in the electrophoretic cell where a potential of $\pm$/150 mV was established. Each batch was analyzed in triplicate [83,102,103].

### 2.2.15 *In vitro* Release Study

A known quantity of Panomycocin loaded CS-TPP NPs powder was dispersed in 10 ml of 100 mM sodium-acetate buffer (pH 5.5) under agitation of 100 rpm and constant temperature of 30 $^{o}$C in order to assess sink conditions during the release studies. At predetermined time intervals (1, 2, 3, 4, 5, 6, 8, 12, 24, 48 h) 10 µl solution was drawn and replaced by an equal volume of fresh medium. Protein concentrations were determined with UV-Visible spectrophometer at 280 nm. The blankCS-TPP NPs were analyzed by the same method to act as controls.All measurements were performed in triplicate [105-107].

### 2.2.16 Antifungal Susceptibility Testings of the Dermatophyte Strains

#### 2.2.16.a Broth Dilution Assay

Dermatophyte (6 *in toto*) strains were tested for their susceptibility to the Panomycocin loaded chitosan-TPP NPs. The Clinical and Laboratory Standards Institute (CLSI; formerly National Committee for Clinical Laboratory Standards, or NCCLS)broth microdilution susceptibility method (M38-A2 Document; Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi) [108] was used for the dermatophyte strains with modifications to ensure the stability of the K5 type yeast killer protein during testing.

Minimum inhibition concentrations (MICs) of the Panomycocin-loaded CS-TPP NPs for dermatophyte strains were also determined by a broth microdilution test using 96 well flat-bottom microtitre plates (Cellstar, Greiner Bio-one, Germany). All dermatophyte strains were grown on PDA agar in glass tubes at 25 $^{o}$C for 7 days in order to provide spore formation. Seven days old colonies were covered with 2 ml sterile saline solution and scratched with tip of a transfer pipette to suspend the spores. Resulting spore suspension was transferred into a sterile tube and allowed to settle down for 10 minutes. After the heavy particles such as hyphal filaments settled down, 1 ml of the upper homogenous suspension was transferred to a sterile tube and vortexed (Heidolph, Germany) for 15 seconds. Spores were counted with a cell-counting haemocytometer to adjust the required cell density of 4 x $10^5$to 25 x $10^5$ spores/ml. Adjusted spore suspensions were further diluted 1:50 in 2 fold concentrated RPMI (pH 5.5) to obtain the 2 x the density needed of 0.4 x $10^4$ to2.5 x $10^4$ spores/ml because the solutions would become a 1 : 2 dilution after the samples were mixed with inoculum. 100 µl of the spore suspension was transferred to the wells of a microtitre plate containing 100 µl of Panomycocin-loaded nanoparticle solution dissolved in acetate buffer solution (pH 5.5) at concentrations of 1 mg/ml, 2mg/ml, 3 mg/ml, 4mg/ml, 5 mg/ml and 6 mg/l to obtain final concentrations of 0.5 mg/ml, 1 mg/ml, 1.5 mg/ml, 2 mg/ml, 2.5 mg/ml and 3 mg/ml. Each row on the microtitre plate also included protein free growth control and sterility control wells. Microtitre plates were incubated at 28$^{o}$C for 2 days and first 6 hours microtitre plates was places on shaker. The MIC endpoints were determined visually at the end of the incubation at 12 h intervals for 48 h with the aid of magnifying mirror by comparing the growth inhibition in each well with that of the growth control well. MIC-0values which corresponds to the lowest nanoparticle concentration producing a clear well or 100% growth inhibition were determined visually at the end of incubation. The experiments were replicated twice for each fungal strain.

#### 2.2.16.b Growth Inhibition of Panomycocin-loaded CS-TPP NPs in a Plate Test

Dermatophyte (6 *in toto*) strains were also tested for their susceptibility to the Panomycocin loaded CS-TPP NPs by growth inhibition in a plate test.Six PDA agar plates (pH 5.5)were assigned to 6 different dermatophytes and they were spread evenly with the inoculum of respective dermatophyte ($1\times10^6$ to $5\times10^6$ spores/ml). After agar absorbs the excess moisture, 10 mg Panomycocin-loaded CS-TPP NPs were placed at the center of agar plates. Then plates were incubated at 28 $^{o}$C for 3-5 days for clear zones to become visible.

# CHAPTER 3

# RESULTS AND DISCUSSION

## 3.1 Production of Crude Killer Toxin

K5 type killer toxin was produced and isolated according to Izgü and Altınbay (2004). *P. anomala* cells were grown in YEPD (pH 4.5) medium with the addition of 5% glycerol as toxin stabilizer at 20 $^{o}$C to maintain the highest degree of killing activity [32] since the productions of the killer toxins are highly dependent on the pH of the cultivating medium and incubation temperature.

## 3.2 Determination of Killer Toxin Activity

Killing activity of the crude protein was tested with agar diffusion assay. Fifty µl of the protein was spotted onto YEPD (pH 4.5) agar seeded with killer toxin sensitive *S. cerevisiae* cells at a cell density of a 0.5 McFarland standard($1\times10^{6}$ to $5\times10^{6}$ cells/ml). After 24 h incubation at 25 $^{o}$C, a clear growth inhibition zone of 22 mm was observed (Figure 3.1).

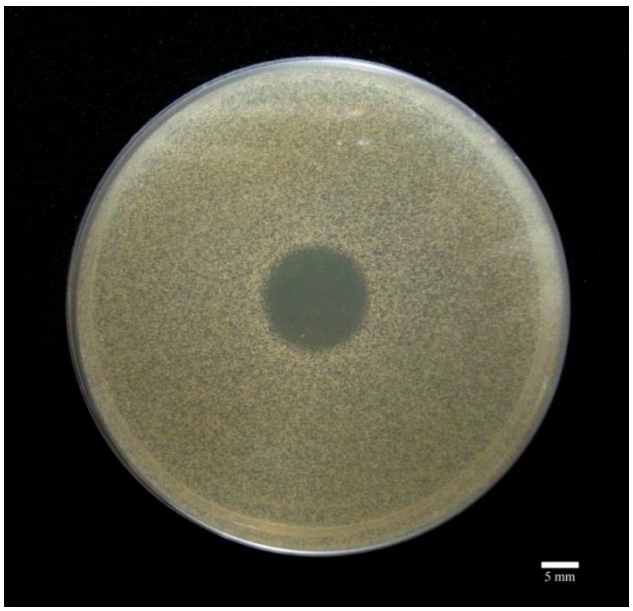



**Figure 3.1** Killer activity of Panomycocin (50 µl) determined by agar diffusion assay.

### 3.3 Isolation of the K5 Type Yeast Killer Toxin

*P. anomala* cells were removed from the culture liquid by centrifugation and concentrated 50 fold by ultrafiltration systems. Concentrated killer protein put on a gel permeation column TSK G 2000SW (Particle size 10µm, Pore size 125 Å, Sample MW 5000 - 100,000 Da) (Figure 3.2). Active fraction was eluted at 8.5 ml (indicated by arrow).

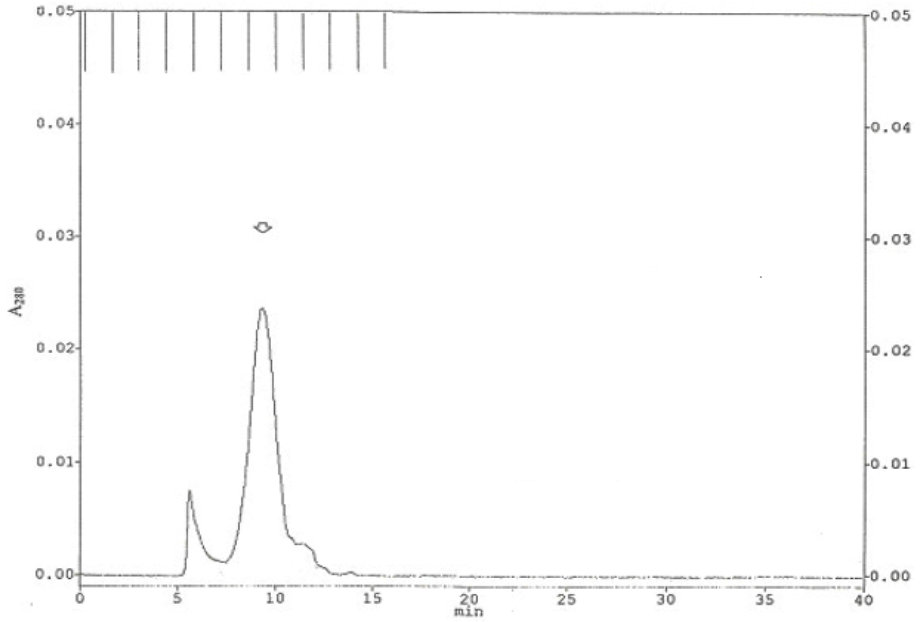



**Figure 3.2** Elution profile of Panomycocin on a TSK G2000SW column. Column size:7.5 mmD / 300 mmL; Sample: 90 µl; Elution buffer: 0.1M $Na_2HPO_4$ pH 4.5 + 0.1 M $Na_2SO_4$; flow rate 1 ml/min; detection 280 nm UV Fraction volume 1300 µl. Fraction containing killer protein is eluted at 8.5 ml and indicated by arrow.

After several runs, active fractions were collected and concentrated by using 5 kDa molecular weight cut off ultrafilters (Vivaspin VS2021, Sartorius, AG, Germany) to a desired protein concentration.

### 3.4 Assessment of Protein Concentration

Final protein concentration was determined by both Bradford method and Nanodrop 2000 UV-Vis spectrophotometer at a wavelength of 280 nm and found to be 1 mg/ml.

### 3.5 SDS Polyacrylamide Gel Electrophoresis

Isolated killer toxin (10 µg) was electrophoresed on a 12.5% linear SDS-PAGE gel in a discontinuous buffer system under denaturing conditions to check its purity. Observation of single protein band on the coomassie brilliant blue stained gel indicates the absence of any contamination and the protein band between markers d and e was confirmed to be the band that belongs to Panomycocin (49 kDa) (Figure 3.3).

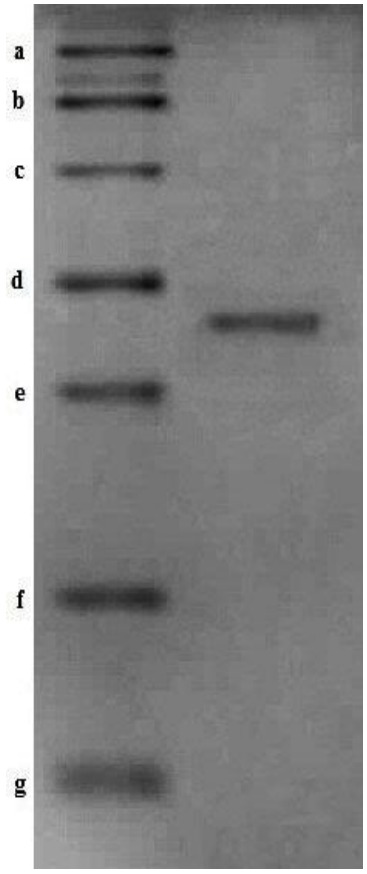



**Figure 3.3** Denaturated SDS-PAGE. Lanes 1 is molecular mass markers (Da) and lane 2 is K5 type yeast killer protein. a) $\alpha_2$-acroglobulin (170,000), b) β-galactosidase (116,353), c) fructose-6-phosphate kinase (85,204), d) glutamate dehydrogenase (55,562), e) aldolase (39,212), f) triose phosphate isomerase (26,626), g) trypsin-inhibitor (20,100).

### 3.6 Preparation of Blank and Panomycocin-loaded Chitosan-TPP NPs

Chitosan and Panomycocin solutions are adjusted to pH 4.5 so that chitosan ($pK_a$~6.5) becomes positively charged and Panomycocin (pI 3.7) becomes negatively charged. According to protein association studies done with differentpH values indicates that thehigher loading capacity is achieved when theprotein is positively charged (when dissolved at a pH above its isoelectricpoint)to render the macromolecule predominantlynegatively charged. Thus,chitosan and protein become oppositely charged leading to a spontaneous forming of nanoparticles, which are then further improved by adding tripolyphosphate[45,77]. The chitosan to TPP weight ratio of 5:1 was confirmed as a stable and suitable ratio to carry out the following analyses of stability and composition,

as done by Calvo et al. [100]. In effect, a 5:1 chitosan to TPP ratio is high enough to observe a colloidal system, but not too high as to drag the zeta potential of the particles too low. In addition, chitosan/TPP volume ratio 2.5:1 was chosen since it has been mostly used in studies, both weight and volume ratios of chitosan/TPP were applied in studies and confirmed to give best efficiency for nanoparticles [74-90,94,100].

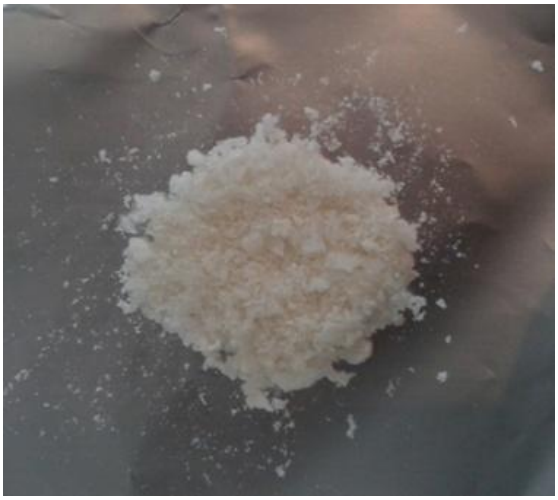


**Figure 3.4** Freeze-dried Panomycocin-loaded CS-TPP nanoparticles.

### 3.7 Evaluation of Association Efficienyand Loading Capacity

After centrifugation of protein loaded CS-TPP nanoparticles at 25000 xg, 4 $^{o}$C, 45 min, supernatant (~26 ml) was taken. Protein concentration in supernatant was determined with UV-Vis spectrophotometer (Nanodrop 2000, Thermo Scientific, USA), absorbance at 280 nm and found as 0.021 mg/ml. Total protein amount in supernant was calculated as 0.55 mg. According to equations given below%AE and %LC were found to be 45 and 0.45, respectively.

$$\textbf{\% AE} = 100 \times \frac{\textbf{Total protein amount} - \textbf{Free protein amount}}{\textbf{Total protein amount}}$$

$$\% \text{ AE} = 100 \times \frac{1 \text{ mg} - 0.55 \text{ mg}}{1 \text{ mg}} \times 100 = 45$$

$$\textbf{\% LC} = 100 \times \frac{\textbf{Total protein amount} - \textbf{Free protein amount}}{\textbf{Weight of nanoparticles}}$$

$$\% \text{ LC} = 100 \times \frac{1 \text{ mg} - 0.55 \text{ mg}}{100 \text{ mg}} = 0.45$$

Wang et al. [82] studied the association efficiency and loading capacity of estradiol (MW 272 Da) as 63.2 and 1.9, respectively. Also, Aktaş et al. [109] reported that %AE and %LC of caspase inhibitor peptide (MW 668 Da) as 12.6 and $0.25\times10^{-5}$, respectively.

Jarudilokkul et al. [79] examined the encapsulation efficiency of fibrinogen (MW 76-81 kDa), transferrin (340 kDa) and BSA (55 kDa) and found as 32.25, 48.21 and 59.46, respectively. In this study, various protein concentrations was tested to improve association efficiency and no linear correlation between the %AE or%LC with the initial protein concentration was found.Tested increasing protein concentration decreased association efficiency probably because more protein cannot be loaded chitosan-TPP nanoparticles and decreasing protein concentration also decreased association efficiency because less protein was loaded in chitosan-TPP nanoparticles. Xu et al. [86] and Jarudilokkul et al. [79] also reported that increasing protein concentration reduced encapsulation efficiency.

### 3.8 Surface Morphology and Particle Size ofBlank CS-TPP NPs and Panomycocin-loaded CS-TPP NPs

The morphological characters of non-crosslinked chitosan, protein-free chitosan-TPP nanoparticles and Panomyocin loaded chitosan-TPP nanoparticles are shown in Figure 3.5. It can be seen that blank (protein-free) non-crosslinked chitosan nanoparticles do not exhibit a smooth surface and had wrinkles on their surface (Fig 3.5.a). Blank CS-TPP nanoparticles are roughly spherical (Fig 3.5.b) and Panomycocin-loaded CS-TPP nanoparticles exhibited a solid dense structure with smooth spherical shape (Fig 3.5.c.d).

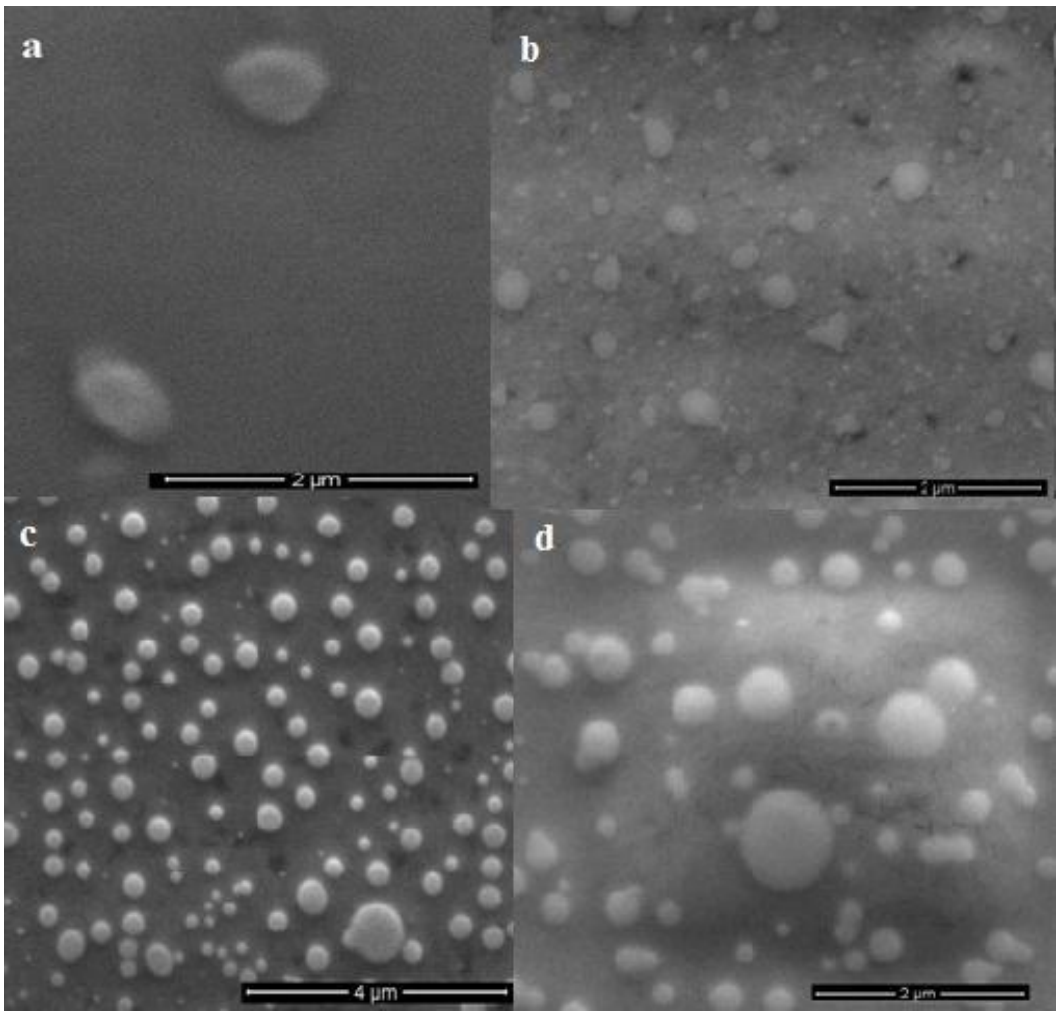



**Figure 3.5** SEM images of(a) Non-crosslinked chitosan (b) Blank CS-TPP NPs, (c,d) Panomycocin-incorporated CS-TPP NPs.

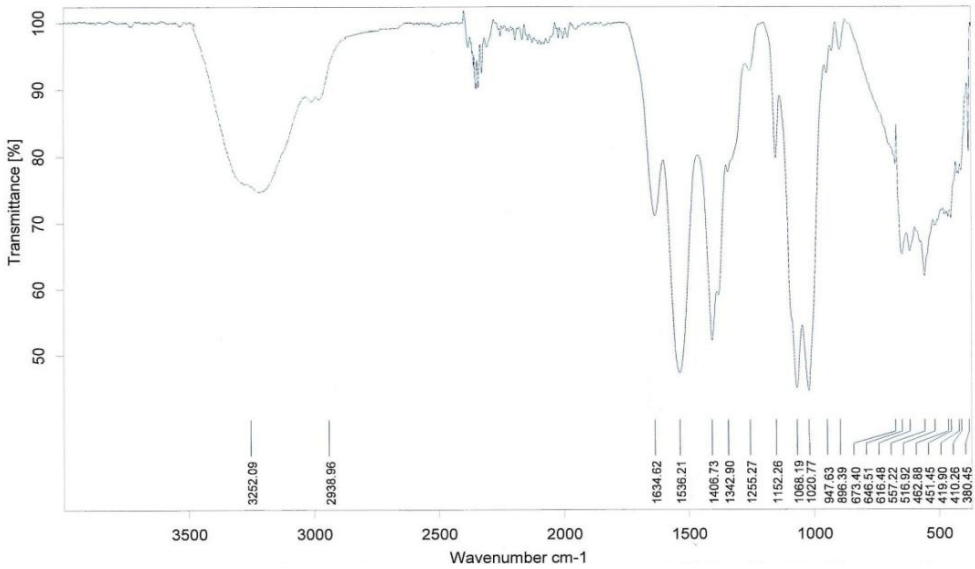
Average particle sizes of blank CS-TPP nanoparticles and Panomycocin-loaded CS-TPP nanoparticles were found to be 230 nm and 325 nm, respectively.There is an increase in particle size of protein-loaded nanoparticles compared to blank nanoparticles. According to many studies, drug loaded chitosan-TPP nanoparticles has larger particle size and has more smooth and spherical shape than drug-free nanoparticles. The reason is postulated that as a result of protein incorporation to chitosan solution, the protein anion occupies the cation of the chitosan and therefore influences the interaction between chitosan polymer and TPP [110].Dounighi et al. [111] examined the particle size of chitosan nanoparticles and *M. eupeus* venom loaded chitosan nanoparticles and found respective average diameters as 260 nm and 370 nm and concluded that venom-loaded nanoparticles are larger than the chitosan-TPP ones. Also, Azevedo et al. [112]determined particle size of inert and insulin loaded chitosan nanoparticles and found as 260.56 nm and 312.8 nm, respectively and concluded that the insulin loading slightly enlarged the nanoparticles.

### 3.9 Fourier Transform Infra Red Spectroscopy (FTIR)

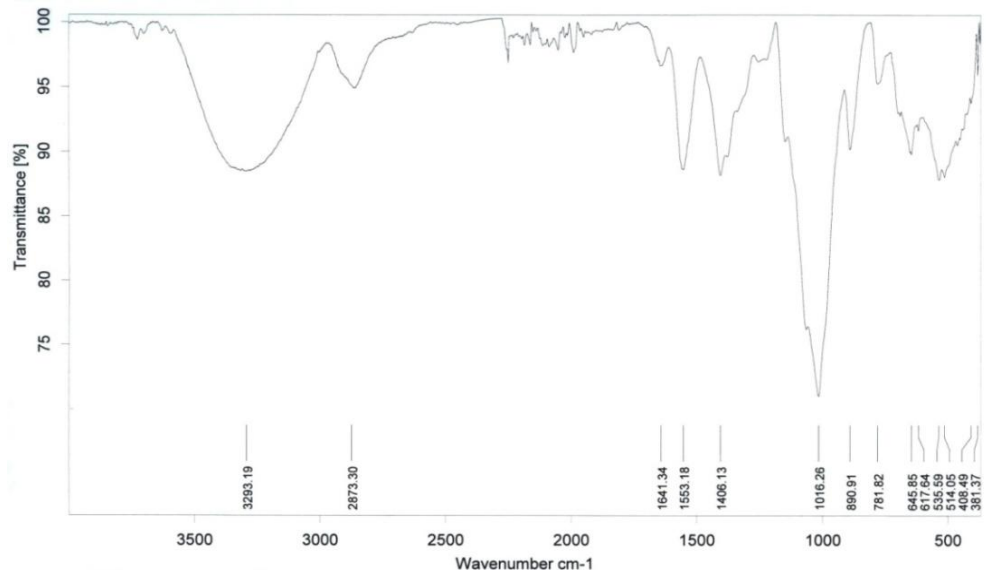
FTIR analyses of nanoparticles were done to identify the presenceof certain functional groups as each of them hasa unique energy absorption band, and to determine the molecular interactions between chitosan, TPP and Panomycocin. Mid-IR range is useful for our purpose which includes wavelength of 2.5 to 25 μm that corresponds to a wavenumber (inverse of wavelength) range of 4000-400$cm^{-1}$range, respectively [105,113].

In order to compare mid-IR spectra of both protein free non-crosslinkedchitosan and TPP crosslinked chitosan nanoparticles (Figure 3.6), in non-crosslinked chitosan IR spectrum, according to Zhang et al. [113], Xu et al. [86] and Dudhania et al. [105]the band at 3252 $cm^{-1}$ corresponds to the combined peaks of stretching vibration of -OH and $–NH_2$groups which are assigned at range of wavenumbers of 3500-3200 $cm^{-1}$ and 3500-3300 $cm^{-1}$, respectively. In TPP-crosslinked chitosan nanoparticles, that peak has a shift to 3293 $cm^{-1}$and becomes wider. This indicates the interaction between these groups and sodium tripolyphosphate and also due to hydrogen bonding indicating that hydrogen bonding is enhanced [86,105,111].For Panomycocin-loaded nanoparticles, this peak shifts to 3303 $cm^{-1}$ and shows more broadening than the CS-TPP NPs, indicating enhanced hydrogen bonding in Panomycocin loaded nanoparticles due to interaction of protein with free amino group of chitosan [84,105].In non-crosslinked chitosan there are absorption bands at 1068 $cm^{-1}$ (anti-symmetric stretch C–O–C) and 1020 $cm^{-1}$(vibrations involving the C–O stretch) which characteristic of chitosan structure [84,105,112].The bands at 1342 $cm^{-1}$ and 1406 $cm^{-1}$ can be assigned to $CH_3$ symmetrical deformation modeand 2938$cm^{-1}$ indicates the –CH stretching vibrations [105,112].In both blank and Panomycocin-loaded CS-TPP NPs, the presence of the –P=O groups of polyphosphate anion at the frequency of 1016 $cm^{-1}$and 1015 cm−1, respectively can be seen [111,112]. The intense peaks for the amide II carbonyl stretch at 1634 $cm^{-1}$and for N-H bending vibration of amide I at 1536 $cm^{-1}$ confirmed the presence of amide I and amide II in the chemical structure of CS [84,105]. After ionotropic crosslinking with TPP, the peaks shifted to 1641$cm^{-1}$and 1553 $cm^{-1}$, respectively anddecreased dramatically, confirming that the strong ionic interaction between positively charged CS and TPP occurs and amino groups were involved in crosslinking by phosphate [105]. Also, in FTIR spectrum of Panomycocin loaded CS-TPP NPs, these peaks slightly shifted to 1645 $cm^{-1}$ and 1559 $cm^{-1}$and band intensities were decreased. This could be related to interactions between Panomycocin and chitosan-TPP NPs such as reported by Azevedo et al. [112] studying interactions between CS-TPP NPs and insulin. Thus, it is confirmed that phosphoric groups of TPP interact with the ammonium groups of chitosan, which enhance both the inter and intramolecular interaction in chitosan nanoparticles.

**a)**




**b)**




**c)**

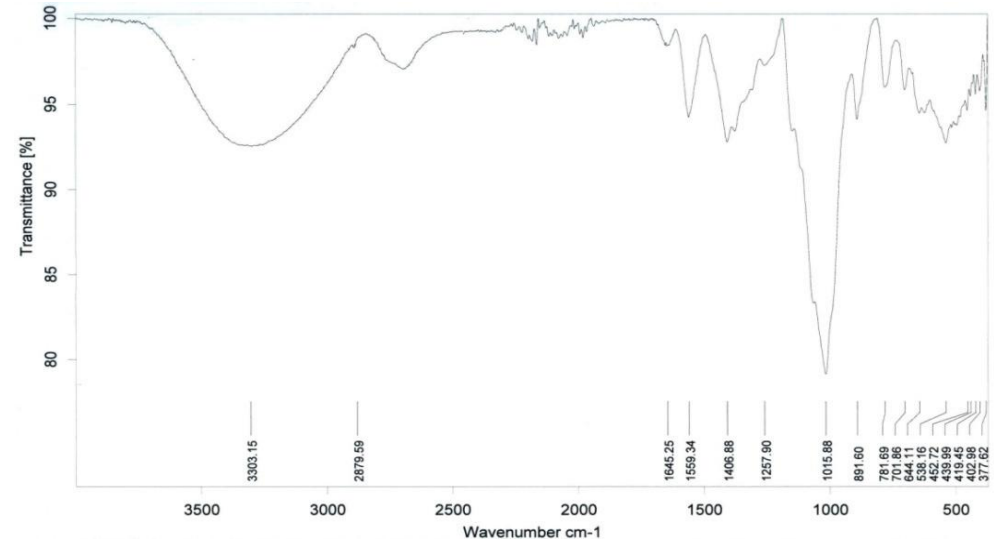



**Figure 3.6** FTIR spectra of (a) CS, (b) CS-TPP NPs, (c) Panomycocin-loaded CS-TPP NPs.

### 3.10 Measurement of Zeta Potential of Blank CS-TPP NPs and Panomycocin-loaded CS-TPP NPs

Zeta potential analyses were done to determine the surface charge of both blank and Panomycocin-loaded nanoparticles. The magnitude of the zeta potentialat a particular pH value is helpful to predictthe state of the nanoparticle surface and the colloidal stability indicating the degree of repulsion between neighboring and similarly charged particles in colloidal solution. For very small particles, a high zeta potential (+ or –) means a good stability, that is solution is resistant to aggregation. When zeta potential is low, it means that solution has poor stability and tend to coagulate due to higher attraction of particles than repulsion. Zeta potential values greater than +25 mV or less than -25 mV confer nanoparticles high degrees of stability [114,115].

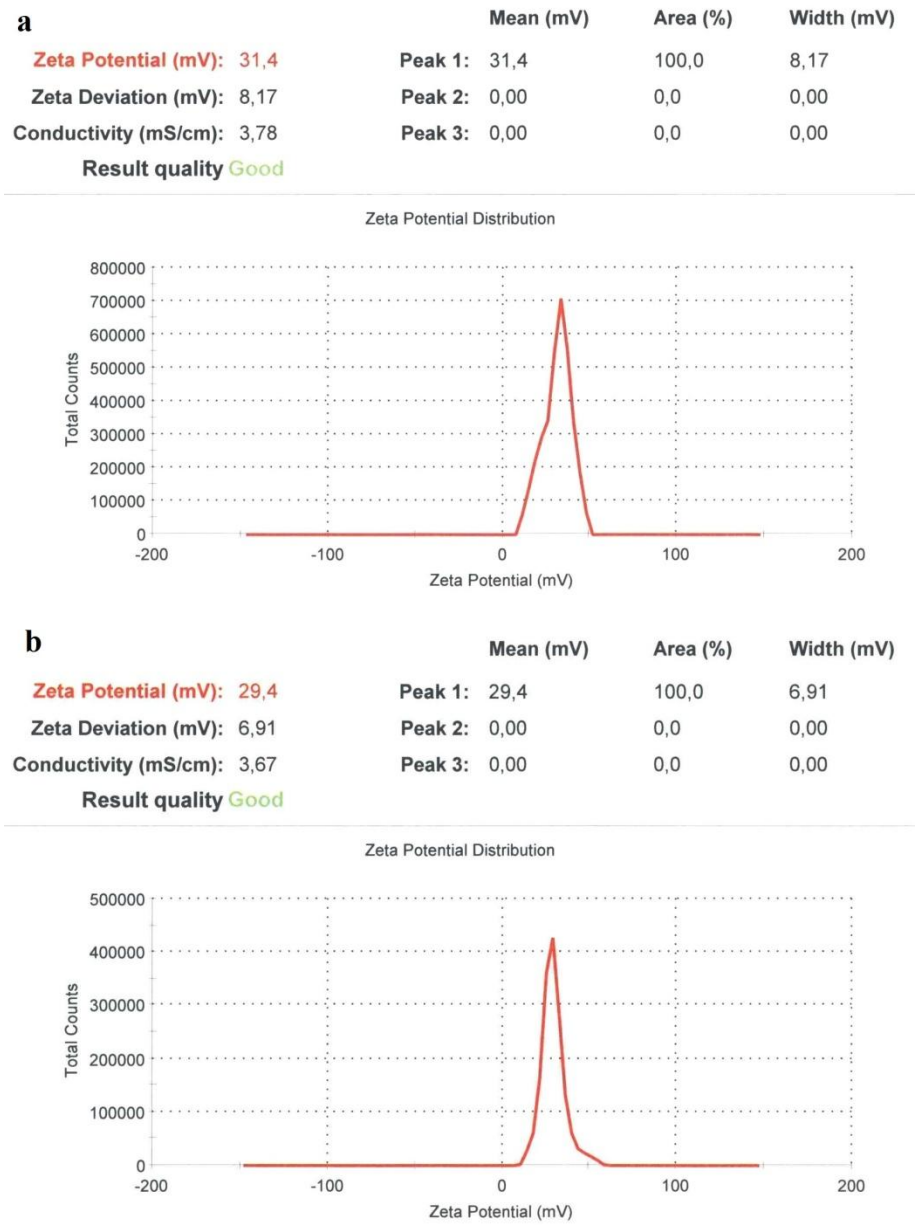



**Figure 3.7** Zeta potential values of a) CS-TPP NPs, b)Panomycocin-loaded CS-TPP NPs.

Zeta potential measurements at pH 4.5of Chitosan-TPP NPs was found as 31.4 ± 8.2 mV and Panomycocin-incorporated CS-TPP NPs was found as 29.4 ± 6.9 mV (Figure 3.7), indicating a moderate to high stability nanoparticles. Since chitosan has a positive surface charge CS-TPP nanoparticles have a positive zeta potential, and addition of negatively charged protein, Panomycocin, leads to a slight reduction of zeta potential, probably due to presence of the protein on the nanoparticle surface.

Similar results were obtained by Wang et al. [82] and Azevedo et al. [112]. Wang et al. [82] determined the average zeta potential of estradiol loaded chitosan-TPP nanoparticles as + 25 mV. Azevedo et al. [112] reported that the zeta potential of chitosan/TPP nanoparticles without insulin was 29 ± 3 mV, while nanoparticles with insulin exhibited load of 23 ± 2 mV, suggesting that the reduction in zeta potential was related by the presence of insulin on the surface of the nanoparticles.

The reason behind the slight decrease of zeta potential was explained by Gan and Wang [45]studying loading of BSA into chitosan-TPP nanoparticles and Dounighi et al. [111] studying loading of *Mesobuthus eupeus* scorpion venom into CS-TPP nanoparticles.They both found that protein loading only slightly decreased the zeta potential of the particles. They speculated that the protein is not uniformly involved with the chain of chitosan molecules. Chitosan molecules in solution are in a spread conformation due to electrostatic repulsion force between positively charged amine groups along the molecular chain. The negatively charged carboxyl groups of a protein molecule may form hydrogen bonds with protonated amine groups at certain sites at the spread chitosan chain. However, to keep their inner hydrophobic core, proteins maintain a compact 3D structure without spreading. Thus, protein attachment didn’tadequately suppress the positive surface charge of chitosan, suggesting that a high proportion of free amine groups on the chitosan chain is unoccupied.

### 3.11 *In vitro* release study

Protein release from nanoparticles takes place by severalmechanisms namely, disintegration of polymer matrix, diffusionof protein molecules and desorptionand mostly show biphasic release pattern with an initial burst followed by a sustained release [45,79,101,105]. Due to the difficulty of directanalysis of the nanoparticles, the protein concentration in the supernatant was determined. The release profile of Panomycocin from CS-TPP NPs are shown in Figure. 3.8.

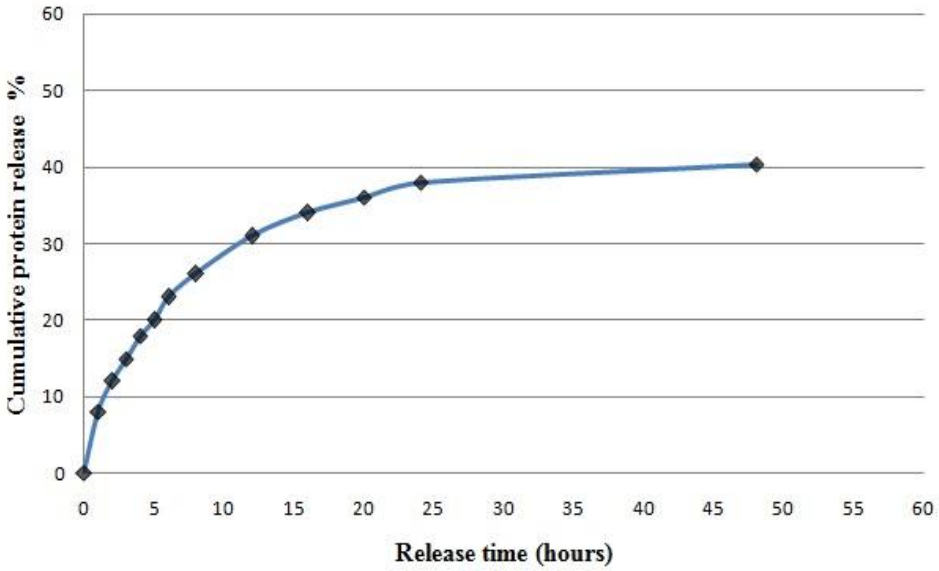



**Figure 3.8** *In vitro* release profile of Panomycocin from CS-TPP NPs.

Panomycocin loaded nanoparticles showed an initial burst release of ~8% which can be the desorption of protein molecules from the nanoparticle surface. According to Xu et al. [86] and Zhou et al. [116], the burst release of protein occurs due to dispersion of protein molecules close to the nanoparticle surface, which can easilydiffuse outduring the initial incubation time. However, since Panomycocin is a large protein (49 kDa), it seems difficult that it can diffuse through thesurface or pores of nanoparticles in a short time as Xu et al. [86] reported the same trend with BSA (MW 55 kDa).The nanoparticles with large surface areacan adsorb Panomycocin; thus, the burst release is possibly due to this part of protein molecules desorbed fromnanoparticles surface. The release of Panomycocin after the initial burst release followed by a slow sustained release.

Over a period of 24 hrs,Panomycocin release reached a plateau at~37% and after 48 hrs, the total protein release was found as ~40%which means there was still some Panomycocin available for further release. Similar results were shown by Vila et al.[117] encapsulating tetanus toxoid (MW 150 kDa) and Jarudilokkul et al. [79] encapsulating transferin (MW 76-81 kDa) and BSA (55 kDa).Since protein and chitosan are oppositely charged, this causes a strong ionic interaction between chitosan and the protein which resulted in less swelling and difficulty of releasing, as FTIR study shows the possible interactions between Panomycocin and chitosan-TPP matrix. Furthermore, because chitosan particles are both bioadhesive and also degradable on the skin and chitosan is able to interact with skin in various mechanisms as mentioned before, Panomycocin could be further released in for prolonged periods following degredation of nanoparticles.

### 3.12 Determination of *In vitro* Antifungal Activity of Panomycocin-incorporated CS-TPP NPs Against Human Dermatophytes

#### 3.12.a Broth Dilution Assay

Susceptibility of 6 dermatophyte strains to the Panomycocin-incorporated CS-TPP NPs was tested with a microtitre plate test. All of the strains including *Microsporum* and *Trichophyton* species were found to be susceptible to nanoparticles.MIC-0 range for *Tricophyton* species was found as 1-2 mg/ml and MIC-0 for *Microsporum gypseum* was found as 1.5 mg/ml.

**Table 3. 1** *In vitro* susceptibility (MIC-0) of 6 dermatophyte strains to Panomycocin-loaded CS-TPP NPs.

| Species | Strain number\Source | MIC-0 (mg/ml) |
|---|---|---|
| *Tricophyton equinum* | DSMZ 122284 | 1.5 |
| *T. interdigitale* | DSMZ 12283 | 2 |
| *T. mentagrophytes* | DSMZ 4870 | 1.5 |
| *T. rubrum* | DSMZ 4167 | 2 |
| *T. tonsurans* | DSMZ 122285 | 1 |
| *Microsporum gypseum* | DSMZ 3824 | 1.5 |

One of the most frequently observed skin diseases is the superficialfungal infections. Especially *Trichophyton* and *Microsporum*species are responsible for the most of the superficial infections. Althoughthe symptoms of these infections are mild and not life threatening, they can actas reservoir of organisms which can spread to other areas of the body or otherindividuals [1-8]. Thus, the susceptibility of dermatophyte strainsincluding *Trichophyton* and *Microsporum* species to the K5 type yeast killertoxin was determined inprevious studies [34] and all of the tested dermathophyte strains were found susceptible to the toxinsince β-1,3-glucanases are known to inhibit germ tube elongation along withsome morphological changes such as leakage of cytoplasm and cell swelling.

Tinea pedis (infection of feet) is one of the most prevalent superficialmycoses caused by *Trichophyton mentagrophytes* or *T. interdigitale* [1,4]. Standard strains of *Trichophyton mentagrophytes* and *Trichophyton interdigitale*were tested against their susceptibility to the K5 type yeast killer toxin and both ofthe strains were found to be susceptible to the toxin at MIC-0 of 2 and 4 μg/mlrespectively. Other strains of the genus including *Trichophyton rubrum, T. equinum* and *T. tonsurans* which are isolated from human skininfection were found to be susceptible to the K5 type yeast killer toxin in theMIC-0 range of 1-4 μg/ml[34]. In this study, susceptibility of these strains to Panomycocin-incorporated CS-TPP NPs was found in the MIC-0 range of 1-2 mg/ml.

*Microsporum gypseum* which are also responsible for tinea infections especially for tinea capitis and tinea corporis werealso tested in this study. MIC-0 value for *M. gypseum* to Panomycocin was found as 2 μg/ml[34]. In this study, MIC-0 for *M. gypseum* was found as 1.5 mg/ml.

### 3.12.b. Growth Inhibition of Panomycocin-loaded CS-TPP NPs in a Plate Test

Dermatophyte (6 *in toto*) strains were also tested for their susceptibility to the Panomycocin loaded chitosan-TPP NPs by growth inhibition in a plate test. Six PDA agar plates were spread evenly with respective dermatophyte inoculum. After agar absorbs the excess moisture, 10 mg of Panomycocin-loaded CS-TPP NPs were placed at the center of agar plates. Then plates were incubated at 28 $^{o}$C for 3-5 days for clear zones to become visible. All six plates have clear zone where nanoparticle powder was seeded (Figure 3.9), indicating high antifungal activity of Panomycocin-loaded CS-TPP nanoparticles.

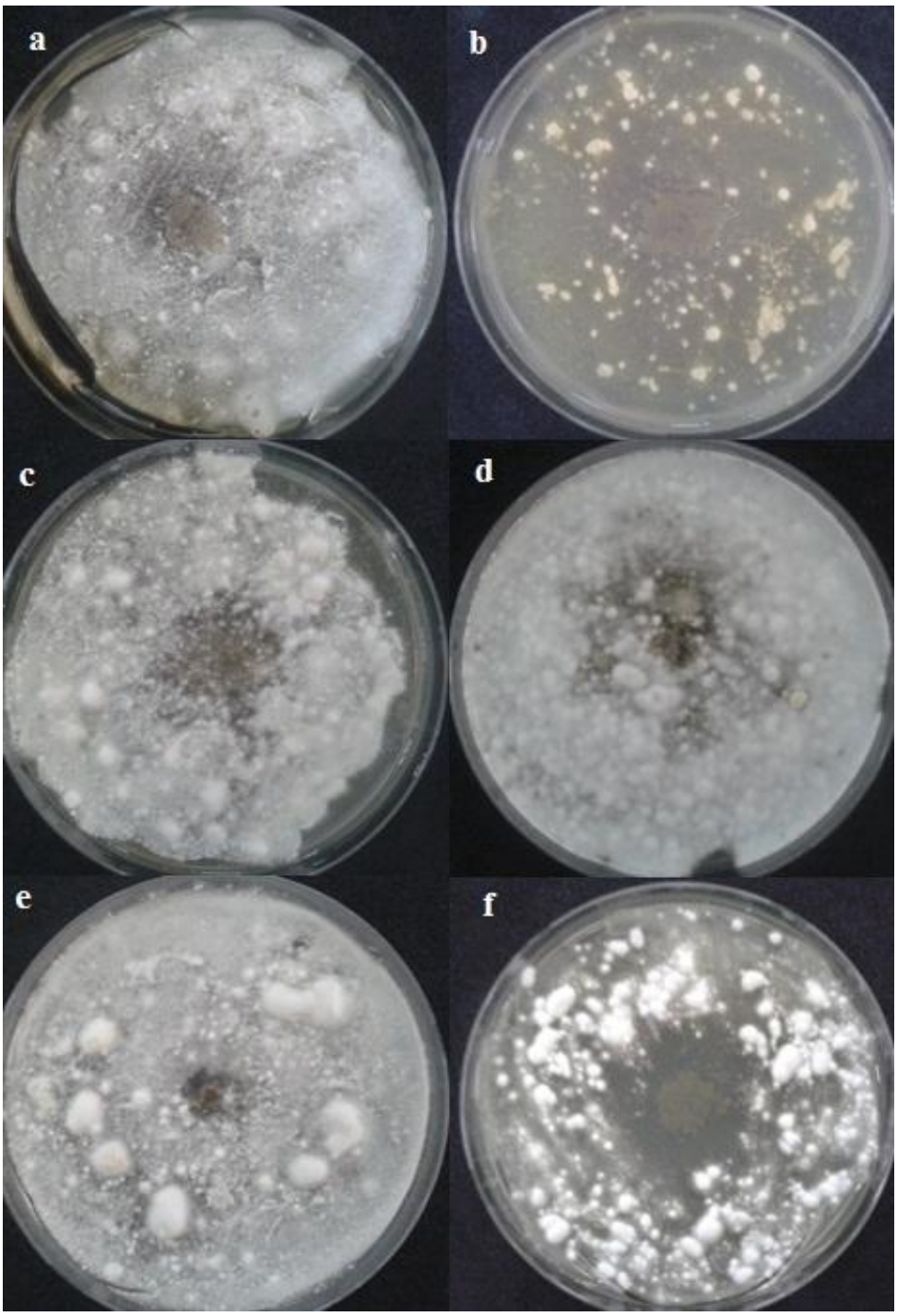



**Figure 3.9** *In vitro* antifungal activity determination of Panomycocin-incorporated CS-TPP NPs (10 mg) against; a)*Tricophyton equinum,* b)*T. interdigitale,* c)*Microsporum gypseum,* d) *T. mentagrophytes,* e) *T. rubrum,*f)*T. tonsurans*by agar diffusion assay.

# CHAPTER 4

# CONCLUSION

This study presents preparation and characterization analyzes of chitosan-TPP nanoparticles loaded with antifungal protein, Panomycocin for the topical treatment of human dermatophyte infections.

Panomycocin-loaded chitosan-TPP nanoparticles were obtained by ionotropic gelation method which is considered a mild and effective process for protein encapsulation into chitosan nanoparticles. The nanoparticles were freeze-dried to obtain powder formulation which provides long shelf-life and easy application to the infected skin.

Panomycocin association efficiency and loading capacity were determined as %45 and %0.45, respectively.

SEM images showed that blank nanoparticles have a roughly spherical shape with wrinkles on their surface with an average particle size of 230 nm and Panomycocin-loaded nanoparticles have a smooth spherical shape with an average particle size of 325 nm.

FTIR studies proved that amino groups of chitosan and phosphate groups of TPP undergo ionic interaction which is needed for nanoparticle formation. Also, analysis of FTIR spectra reveals an interaction between Panomycocin and chitosan-TPP.

Zeta potential measurements were done at pH 4.5 and zeta potential of chitosan-TPP nanoparticles was found as 31.4 ± 8.2 mV while zeta potential of Panomycocin-loaded chitosan-TPP nanoparticles was found as 29.4 ± 6.9 mV, indicating a high stability of nanoparticles and entrapment of Panomycocin into nanoparticles.

*Invitro* release of Panomycocin from chitosan-TPP nanoparticles showed a 40% total release of protein over a period of 48 hours having a biphasic release pattern with an initial burst followed by a slow sustained release.

Broth dilution tests against 6 human dermatophyte strains confirmed that all tested dermatophyte strains were found to be susceptible to Panomycocin-loaded CS-TPP NPs. MIC-0 range for *Tricophyton* species was found as 1-2 mg/ml and MIC-0 for *Microsporum gypseum* was found as 1.5 mg/ml.

As a result of characterizations and antifungal activity determination tests, this study shows that Panomycocin can be successfully incorporated into chitosan nanoparticles and released in the active form. this formulation enables topical antifungal treatment of Panomycocin in powder form against human dermatophyte infections and the results of this study can be studied for further *in vivo* tests and clinical trials.

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## APPENDIX A

## CHEMICALS AND THEIR SUPPLIERS

Acetic Acid (Merck, Germany)
Acrylamide (Boehringer Mannheim, Germany)
Ammoniumpersulphate (Pharmacia Biotech, Sweden)
Bacto-agar (Difco, USA)
Bacto-peptone (Difco, USA)
Bis-acrylamide (Boehringer-Mannheim, Germany)
Butanol (Merck, Germany)
β-mercaptoethanol (Sigma, USA)
Coomassie Brilliant Blue G-250 (ICN, USA)
D-Glucose (Merck, Germany)
Di-potassium Hydrogen Phosphate (Merck, Germany)
Ethanol (Merck, Germany)
Glycerol (Merck, Germany)
Hydrochloric Acid (Merck, Germany)
Lactose (Merck, Germany)
Malt extract (Difco, USA)
PDA (Merck, Germany)
Potassium Dihydrogen Phosphate (Merck, Germany)
Sodium Carbonate (Merck, Germany)
Sodium Chloride (Merck, Germany)
Sodium Dodecyl Sulfate (Merck, Germany)
Sodium Hydroxide (Merck, Germany)
Sodium Sulfate (Merck, Germany)
TEMED (Pharmacia Biotech, Sweden)
Trichloroacetic Acid (Merck, Germany)
Tris (Merck, Germany)
Yeast extract (Difco, USA)

## APPENDIX B

## BUFFERS AND SOLUTIONS

**Table B.1 SDS PAGE Gel Components and Staining Solutions**

| Buffers/ Solutions | Composition |
|---|---|
| **1.SDS-PAGE** | |
| Monomer Solution | 30.8% T, 2.7% $C_{bis}$ |
| 4X Running Gel Buffer | 1.5 M Tris-Cl, pH 8.8 |
| 4X Stacking Gel Buffer | 0.5 M Tris-Cl, pH 6.8 |
| SDS | 10% |
| Initiator | 10% Ammonium Persulfate |
| 2X Treatment Buffer | 0.125 M Tris-Cl, 4% SDS, 20% Glycerol, 10% β-mercaptoethanol, 0.020% Bromophenol blue, pH 6.8 |
| Tank Buffer | 0.025 M Tris, 0.192 M Glycine, 0.1% SDS , pH 8.3 |
| **2.COOMASSIE STAIN** | |
| Staining Solution | 0.025% Coomassie Brilliant blue R 250, 40% methanol, 7% acetic acid |
| Destaining Solution I | 40% methanol, 7% acetic acid |
| Destaining Solutiın II | 7% acetic acid, 5% methanol |

**Table B.2 RPMI 1640 Components**

| RPMI 1640 COMPONENTS | Conc. (mg/L) |
|---|---|
| **INORGANIC SALTS:** | |
| Calcium nitrate (Ca(NO3)2 4H2O) | 100.00 |
| Potassium chloride (KCl) | 400.00 |
| Magnesium sulfate (MgSO4 ) | 48.84 |
| Magnesium sulfate (MgSO4 7H20) | 100.00 |
| Sodium chloride (NaCl) | 6000.00 |
| Sodium Phosphate (Na2HPO4) | 800.00 |
| **OTHER COMPONENTS:** | |
| Glucose | 2000.00 |
| Glutathione Reduced | 1.00 |
| Phenol red | 5.00 |
| **AMINO ACIDS:** | |
| L-Arginine | 200.00 |
| L-Asparagine | 50.00 |
| L-Aspartic Acid | 20.00 |
| L-Cystine dihydrochloride | 65.00 |
| L-Glutamic Acid | 20.00 |
| L-Glutamine | 300.00 |
| Glycine | 10.00 |
| L-Histidine | 15.00 |
| L-Hydroxyproline | 20.00 |
| L-Isoleucine | 50.00 |
| L-Leucine | 50.00 |
| L-Lysine hydrochloride | 40.00 |
| L-Methionine | 15.00 |
| L-Phenylalanine | 15.00 |
| L-Proline | 20.00 |
| L-Serine | 30.00 |
| L-Threonine | 20.00 |
| L-Tryptophan | 5.00 |
| L-Tyrosine disodium, dihydrate | 29.00 |
| L-Valine | 20.00 |
| **VITAMINS:** | |
| Biotin | 0.2 |
| D-Ca Pantothenate | 0.25 |
| Choline Chloride | 3.00 |
| Folic Acid | 1.00 |
| i-Inositol | 35.00 |
| Niacinamide | 1.00 |
| p-Aminobenzoic Acid (PABA) | 1.00 |
| Pyridoxine HCl | 1.00 |
| Riboflavin | 0.20 |