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RESEARCH PAPER

Fast-Acting Clotrimazole Composited PVP/HPβCD Nanofibers for Oral Candidiasis Application

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ABSTRACT

Purpose This study investigates fabrication of clotrimazole (CZ)-composited electrospun Polyvinylpyrrolidone/Hydroxypropyl-β-cyclodextrin (PVP/HPβCD) blended nanofiber mats for oral candidiasis applications.

Methods PVP/HPβCD blended nanofiber mats containing clotrimazole were electrospun and characterized using SEM, DSC and XRPD. The solvent system ethanol: water: benzyl alcohol (EtOH:H_2O:BzOH) with a 70:20:10 ratio was optimal for the electrospinning process. Various amounts of CZ were loaded into the nanofiber mats. The nanofiber mats was further investigated for drug release, antifungal activity and cytotoxicity.

Results The fiber diameters in the mats were in the nanometer range. The DSC and XRPD revealed a molecular dispersion of amorphous CZ in the nanofiber mats. The loading capacity increased when CZ content was raised. A fast dissolved and released of CZ from the nanofibers mat was achieved. The ability of the CZ-loaded nanofiber mats to kill the *Candida* depended on the amount of CZ in the mats; moreover, the CZ-loaded nanofibers killed the *Candida* significantly faster than the CZ powder and lozenges with low cytotoxicity.

Conclusions CZ-loaded nanofiber mats were successfully electrospun. They exhibited rapid antifungal activity *in vitro* relative to CZ powder and lozenges. Further *in vivo* studies are needed to investigate for their application in oral candidiasis.

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INTRODUCTION

Candida spp. may cause opportunistic infections in patients both local and systemic risk factors; these types of infections and are extremely common with individuals with human immunodeficiency virus (HIV) and immunocompromised patients (1,2). Incidences of oropharyngeal infection by *Candida spp.* or oropharyngeal candidiasis (OPC) are increasing worldwide; infections due to *Candida albicans* are more common (3,4). The clinical symptoms of oropharyngeal candidiasis can be odd sensations, altered taste, and altered smell, affecting the patient's quality of life (5,6). Numerous effective antifungal drugs may be used either topically or systemically to manage OPC, but the most efficient treatment are classical polyenes, and azole group antimycotics which are classified into imidazoles and triazoles (7,8). The azoles are becoming increasingly popular in OPC therapy.

Clotrimazole (CZ) is a lipophilic broad-spectrum antimycotic drug utilized to treat fungal infections (9,10) and is mainly used to manage superficial candidiasis in the oral cavity, vagina and skin. Nevertheless, it has poor aqueous solubility (0.49 μg/ml) (11) that might affect its antimycotic activity. The topical application of CZ into the oral cavity must be washed off rapidly to increase patient compliance. Therefore, enhancing the solubility and release rate of CZ is necessary for rapid antimycotic activity.

Recently, new strategies for drug delivery have gained increasing interest. Fast-dissolving drug delivery systems are important because they have numerous advantages, such as enhanced drug solubility, onset of action and bioavailability (12,13). To achieve the fast-dissolution, the selection of appropriate polymer that can dissolve rapidly and easily is

necessary. Polyvinylpyrrolidone (PVP) can provide these features; it is capable of ultrafast dissolution. Cyclodextrins (CDs) are cyclic oligosaccharides composed of 1,4-linked glucopyranoside units that can form inclusion complexes with a broad range of drug molecules, altering the physicochemical properties of the complexes (14). Complexation of poorly soluble drugs with cyclodextrin can improve the dissolution and stability of the drugs (15–17). Previous studies indicate that blending hydroxypropyl-β-cyclodextrin (HPβCD) with PVP can improve the physical properties of PVP; in particular, this mixture might reduce the hygroscopicity of PVP (18–20). Moreover, a cyclodextrin inclusion complex reduces the unpleasant odor and taste of the drug by encapsulating it at the molecular level. Therefore, blending HPβCD with PVP is of particular interest.

Nanofiber based scaffolds have potential applications in many fields (tissue engineering, drug delivery systems, wound dressing, filtration etc.) because nanofibers exhibit outstanding characteristics, such as low density, high porosity, large specific surface areas and very small pore sizes (21–23). Electrospinning has been intensively studied and is one of the most promising processes for producing nanofiber scaffolds due to its efficiency, simplicity in fabrication, versatility, low cost and potential to be scaled up to industrial levels (24,25). In this work, CZ-loaded electrospun nanofiber mats using HPβCD functionalized PVP to form the polymeric filaments were developed. The properties of the electrospun nanofibers and drug release characteristics were investigated. Moreover, the nanofiber mats were also investigated for antifungal activity and cytotoxicity.

MATERIALS AND METHODS

Materials

Clotrimazole (CZ), 2-hydroxypropyl-β-cyclodextrin (HPβCD), Polyvinylpyrrolidone (PVP, MW. ~1,300,000) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Chemical Co. (St. Louis, MO, USA). Sabouraud dextrose broth was purchased from Becton, Dickinson and Company (Franklin Lakes, NJ, USA). The human gingival fibroblasts (HGF) were obtained from the Faculty of Dentistry, Naresuan University, Thailand. Dimethyl sulfoxide (DMSO) was obtained from BDH Laboratories, UK. Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA, penicillin-streptomycin antibiotics and fetal bovine serum (FBS) were obtained from GIBCO-Invitrogen (Grand Island, NY, USA). All other reagents and solvents were of analytical grade and were used without further purification.

Solubility Studies

The solubility of CZ in various solvents was determined using a shaken flask method. Excess CZ was added to a plastic eppendorf tube containing the selected vehicle and mixed using a vortex mixer. Mixtures were shaken for 24 h in an orbital shaking incubator (Comecta S.A., Abrera, Spain) maintained at 37°C before being centrifuged at 10,000 rpm for 10 min, and filtered through a 0.45 μm syringe driven membrane filter unit (VertiClean^{TM} Nylon Syringe filters,13 mm, 0.45 μm). The filtrates were assayed to determine the amount of dissolved drug using an HPLC (Agilent Technologies, USA) with a detector operating at 215 mm and a Phenomenex® C18 column (150×4.60 mm, 5 μm particle size) with a C18 guard column. The elution was carried out using a solvent system composed of methanol and ammonium carbonate buffer solution (75:25) at 2 ml/min. The experiments were performed in triplicate.

Phase Solubility Studies

Phase solubility studies were carried out according to the method described by Higuchi and Connors (26). Excess CZ was mixed in an aqueous solution, and solvent system containing EtOH: H_2O: BzOH in a 70:20:10 ratio (by volume) containing increasing amounts of HPβCD (30, 50, 70, 90, 110, 130 and 150 mM) and agitated on a horizontal movement shaker (30 rpm) at 37°C until equilibrium was reached (24 h). After equilibrium, the CZ concentration remained the same, samples were collected and centrifuged, and their CZ content was quantified using HPLC. The experiments were carried out in triplicate.

Electrospinning of CZ-Loaded PVP/HPβCD Nanofibers

Ethanol (EtOH) and benzyl alcohol (BzOH) were chosen because they are good solvents for both PVP and CZ and relatively non-toxic. To determine the optimal amount of BzOH for the electrospinning process, 8% PVP and 70 mM HPβCD were dissolved in EtOH: H_2O: BzOH with volume ratios of 70:30:0, 70:25:5 and 70:20:10. After determining the optimal BzOH content, Clotrimazole (5%, 10%, 15% and 20%wt to polymer) was added to the mixture and stirred for 12 h at room temperature. The viscosity and conductivity of these mixed solutions were determined using a Brookfield viscometer (DV-III ultra, Brookfield Engineering Laboratories, USA) and a conductivity meter (Eutech Instruments Pte Ltd, Singapore), respectively. The spinning solution was placed in a 5 ml glass syringe connected to a stainless steel needle with a 0.9 mm inner diameter. The needle was connected to an emitting electrode with a positive polarity of a Gamma High Voltage Research device. The electrospinning process was conducted at 25°C with a fixed

applied voltage of 15 kV, a distance between the tip and the collector of 15 cm, and a feeding rate of 0.3 ml/h. The electrospun nanofibers were collected on an aluminum foil covered the rotating collector.

Characterization of CZ-Loaded PVP/HPβCD Nanofibers

Scanning Electron Microscope (SEM)

The morphology and diameter of the nanofiber mats was examined using a scanning electron microscope (SEM, Camscan Mx2000, England). For this process, a small section of the fiber was sputtered with a thin layer of gold before the SEM observations. The average diameter of the fibers was determined using image analysis software (JMicroVision V.1.2.7, Switzerland).

Fourier Transform Infrared Spectrophotometry (FT-IR)

The chemical structure of the fibers was characterized using a Fourier transform infrared spectrophotometer (FT-IR, Nicolet 4700, USA). The fiber samples were ground and pressed into KBr dishes pellets before being analyzed by the FT-IR analysis from 400 to 4,000 cm^{-1}.

Differential Scanning Calorimetry (DSC)

The thermal behavior of the nanofiber mats was determined using differential scanning calorimetry (DSC, Pyris Diamond DSC, PerkinElmer instrument, USA). The experiments were conducted in dry, flowing nitrogen with samples weighing of approximately 5 mg. The DSC traces were recorded from 50 to 250°C at 10°C/min.

Powder X-Ray Diffractometry (PXRD)

Powder X-ray diffraction analyses (PXRD, Miniflex II, Rigaku, Japan) of the samples were carried out to investigate the physical state of the CZ in the nanofiber mats using Nickel-filtered Cu radiation generated in a sealed tube operated at 30 kv and 15 mA. The diffraction patterns of the nanofiber mats were recorded in the θ range of 5–45° at 5^{o} min^{-1}.

Determination of CZ Content

The total CZ content in the CZ-loaded nanofiber mats was determined in triplicate. Accurately weighed samples (1 mg) of the fiber mats were placed in a 1.5 ml plastic eppendorf tubes containing 1 ml of methanol, and continuously shaken in a incubator (Orbital Shaking Incubator Model: SI4) at 150 rpm for 24 h. The CZ content was measured using HPLC. The entrapment efficiency (%) and loading capacity (mg/mg) were calculated according to Eqs. (1) and (2), respectively:

$$\%\ \text{Entrapment efficiency} = (P_t/L_t) \times 100 \quad (1)$$

where P_t is the amount of CZ embedded in the nanofiber mats and L_t is the theoretical amount of CZ (from the feeding solution) incorporated into the nanofiber mats.

$$\text{Loading capacity} = (P_t/M_t) \quad (2)$$

where P_t is the amount of CZ embedded in the nanofiber mats and M_t is the weight of nanofiber mats.

Wetting Time and Disintegration Time

The wetting time study was performed using a procedure modified from the literature (13). Two layers of tissue paper were placed on a Petri dish with a diameter of 10 cm. The nanofibrous membranes were placed on the paper wetted with artificial saliva (2.38 g Na_2HPO_4, 0.19 g KH_2PO_4, and 8 g of NaCl per liter of distilled water adjusted with the phosphoric acid to pH 6.8±0.05) (27), and the excess saliva was completely drained out. For the disintegration study, artificial saliva was used to evaluate the rate of dissolution of the nanofibrous membranes. The wetting and disintegration processes were recorded using a digital video recorder (Cannon, Japan).

In Vitro Release

The *in vitro* release studies were adapted from Singh *et. al.* (28). Briefly, 5 mg of the CZ-loaded nanofiber mats were placed in a 100 ml bottle containing 50 ml of artificial saliva (pH 6.8) containing 20% PEG-400 that was incubated at 37°C while being shaken at 150 rpm. To determine the amount of CZ released from the fiber mats after a given interval, an aliquot (1.0 ml) of the release medium solution was withdrawn and replaced with the same volume of fresh medium to maintain a constant volume. The amounts of CZ in the sample solutions were analyzed by HPLC. The experiments were conducted in triplicate.

Antifungal Activity of the Nanofiber Mats

Bacterial Strains and Culture Conditions

Two *Candida* strains, (*Candida albicans* ATCC 90028 and *Candida dubliniensis* (a clinical oral isolated from HIV-free patients with candididiasis)) were used. The stock cultures were maintained at −80°C. After recovery, this culture was maintained on Sabouraud dextrose agar (SDA) and stored at 4–8°C during the experimental period. To prepare the yeast

inoculum, a loopful of the stock culture was streaked onto SDA and incubated at 37°C for 48 h. A loopful of this culture was transferred to 5 ml of Sabouraud dextrose broth (SDB) medium and incubated at 37°C for 24 h. The *Candida* suspensions were diluted in SDB and spectrophotometrically standardized to 1×10^6 CFU/ml (Perkin Elmer Lambda 2, Germany).

Susceptibility Testing

The antifungal activity of CZ was determined via susceptibility testing and the results were expressed as the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) toward Candida cells. A broth microdilution method was undertaken in accordance with the guidelines recommended by the CLSI (Clinical and Laboratory Standards Institute, 2002) using serial two-fold dilutions of CZ in Sabouraud dextrose medium to determine susceptibility of the *C. albicans* and *C. dubliniensis* toward clotrimazole. Briefly, *C. albicans* and *C. dubliniensis* were incubated (10^4 CFU/ml) in 48-well plates for 48 h at 37°C before being exposed to serial 2-fold dilutions of the SDB culture medium with the CZ solution (from 200 to 0.4 μg/ml). The MIC was defined as the lowest concentration at which there was no visible growth by visual inspection. To establish the MFC, aliquots from the first 3 wells without visible growth by visual inspection were removed and inoculated (100 μl) in SDA for 48 h. The MFC value was defined as the lowest concentration of CZ solution that exhibited no growth. All assays were performed in triplicate.

Time-Kill Analysis

The time kill analyses were performed to determine the exposure time required to kill a standardized microbial inoculum. Briefly, the rate of killing fixed inoculums of *C. albicans* and *C. dubliniensis* (usually 10^4 CFU/ml) was determined by incubating the test organism with agitation in SDB medium containing different concentrations of CZ from nanofiber mats, powder or lozenges (10 mg, Candinas troche; Thailand Jan Laboratories). The 5 mg samples of nanofiber mats with different CZ-loadings (0–20%) were added to a *Candida* suspension. In order to compare the killing rate with CZ powder and lozenges, the 20% CZ loaded nanofiber mats with a CZ content equivalent to 1.6 mg, CZ powder (1.6 mg) and CZ lozenges with a CZ content equivalent to 1.6 mg were added to a *Candida* suspension at the final concentrations of 1.0 μg/mL. Aliquots of control and CZ-containing media were removed and diluted at 5, 15, 30, 60, 120 and 240 min before the surviving colonies were counted (CFU/ml) by spreading each sample onto an SDA agar plate. The plates were incubated for 24 h and the viable colonies were assessed. The kill curves were constructed by plotting the CFU/ml surviving at each time point in the presence and absence of the nanofiber mats with different CZ concentrations.

Evaluation of Cytotoxicity

The cytotoxicities of the CZ and CZ-loaded nanofiber mats were evaluated using an MTT cytotoxicity assay. Human gingival fibroblast cells (HGF) obtained from explants of gingival tissue attached to non-carious, freshly extracted third molars from three patients were used. All patients gave informed consent before tissue collection. Ethical approval for the study was obtained from Naresuan University. The HGF cells were plated in 100 μl of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 2 mM L-glutamine, 1% non-essential amino acids and 0.1% penicillin-streptomycin before being distributed at 10,000 cells/well in 96-well plates. The cells were grown under a humidified atmosphere (5% CO_2, 95% air, 37°C) until confluency (typically 24 h). The cells were treated with CZ at concentrations ranging from 0.1 to 100 μg/ml in a serum-free medium (pH 7.4) and subsequently incubated for 2 and 24 h. For the CZ-loaded nanofiber mats, the cytotoxicity tests were performed based on a procedure adapted from the ISO10993-5 standard testing method (indirect contact). CZ lozenges and CZ (0–20%)-loaded nanofibers were sterilized using UV radiation for 1 h before being immersed in a serum-free medium (SFM; containing DMEM, 1% l-glutamine, 1% lactalbumin and 1% antibiotic and antimycotic formulation) and incubated for 24 h to produce extraction media with varying concentrations. The extraction media at varying concentrations were replaced, and the cells were re-incubated for 2 h and 24 h. After treatment, the serum-free medium containing CZ and the tested extraction solutions were removed. Finally, the cells were incubated with 100 μl of an MTT-containing medium (1 mg/ml) for 4 h. The medium was removed, the cells were rinsed with a phosphate buffer (pH 7.4), and the formazan crystals that formed in the living cells were dissolved in of 100 μl DMSO per well. The relative viability (%) was calculated based on the absorbance at 550 nm determined using a microplate reader (Universal Microplate Analyzer, Model AOPUS01 and AI53601, Packard BioScience, CT, USA). The viability of non-treated control cells was arbitrarily defined as 100%.

$$\text{Relative cell viability}(\%) = \frac{\left[\text{OD}_{550,\text{sample}} - \text{OD}_{550,\text{blank}}\right]}{\left[\text{OD}_{550,\text{control}} - \text{OD}_{550,\text{blank}}\right]} \times 100 \quad (3)$$

Statistical Analysis

All experimental measurements were collected in triplicate. The values are expressed as the means ± standard deviation

Table I Solubility of CZ in Various Solvents. Each Value Represents the mean ± S.D. from Three Independent Experiments

Test solvent	Solubility (mg/ml)
Water	0.000402 ± 0.00021
Ethanol	128.15 ± 12.43
Benzyl alcohol	646.36 ± 66.12
Solvent mixture of EtOH: H_2O: BzOH with volume ratio of 70:20:10	265.23 ± 17.37

(SD). The statistical significance of the differences in each experiment was examined using one-way analysis of variance (ANOVA), followed by a least significant difference (LSD) post hoc test. The differences were significant at $p<0.05$.

RESULTS AND DISCUSSION

CZ Solubility

The solubility of CZ was carried out in water (H_2O), ethanol (EtOH), benzyl alcohol (BzOH) and a solvent mixture (EtOH: H_2O: BzOH at volume ratio of 70: 20: 10), as presented in Table I. CZ exhibited very poor solubility in water (0.402 ± 0.21 μg/ml). This result was similar to a previous study revealing that the solubility of CZ in water was 0.49 μg/ml (11). CZ appeared to have good solubility in ethanol (128.15 ± 12.43 mg/ml) and very good solubility in benzyl alcohol (646.36 ± 66.12 mg/ml). Therefore, benzyl alcohol was chosen as the solvent mixture for electrospinning the CZ-loaded PVP/HPβCD nanofiber mats.

Phase Solubility Study

The phase solubility study might be a beneficial method for examining inclusion complexation because it enhances the solubility ability and the apparent stability constant of the complexes (26). Therefore, the inclusion complexation of CZ and HPβCD was assessed via phase solubility study. The phase solubility diagram for the CZ and CDs systems in aqueous, and EtOH:H_2O:BzOH (70:20:10 by volume) solvent systems at 37°C are depicted in Fig. 1. The inclusion complex displayed a different pattern in the aqueous solution (Fig. 1a) and EtOH:H_2O:BzOH solvent system (Fig. 1b). In the aqueous solution, the solubility of CZ increased linearly with the HPβCD concentration; therefore, it formed an A_L type inclusion complex indicating the complex had a 1:1 ratio. In contrast, the solubility of CZ in the EtOH:H_2O:BzOH (70:20:10) solvent system did not increase linearly as a linear function of HPβCD concentration and thus was identified as a B_S type inclusion complex indicating the formation of a 1:2 complex. CZ was most soluble in the solvent system at 50 mM HPβCD.

Electrospinning of PVP/HPβCD Nanofiber Mats

The CZ-loaded PVP/HPβCD was electrospun in various EtOH:H_2O:BzOH ratios (70:30:0, 70:25:5 and 70:20:10 v/v). The solution parameters before electrospinning are listed in Table II. The amount of BzOH added to the solution affected the morphology of the nanofiber mats, as illustrated in Fig. 2. Increasing in the BzOH content in the mixture resulted in a lowered bead-on-string content; in addition, the fiber became more uniform and grew in diameter due to the enhanced viscosity of the solution. These results were in accordance with a previous study revealing that beaded nanofibers were created from an electrospinning solution with a lower viscosity (29,30). By increasing the BzOH content to 5% v/v, The number of beads was reduced, and the fibers became more uniform; however, the fiber diameter increased due to the solution viscosity, and the conductivity dramatically increased and decreased respectively (Table II). However, the viscosity and conductivity were not significantly different when the BzOH content was increased from 5% to 10% v/v. Therefore, the increase in fiber diameter was not significant. The average fiber diameters of nanofibers that were electrospun from solutions containing 0%, 5% and 10% v/v BzOH content

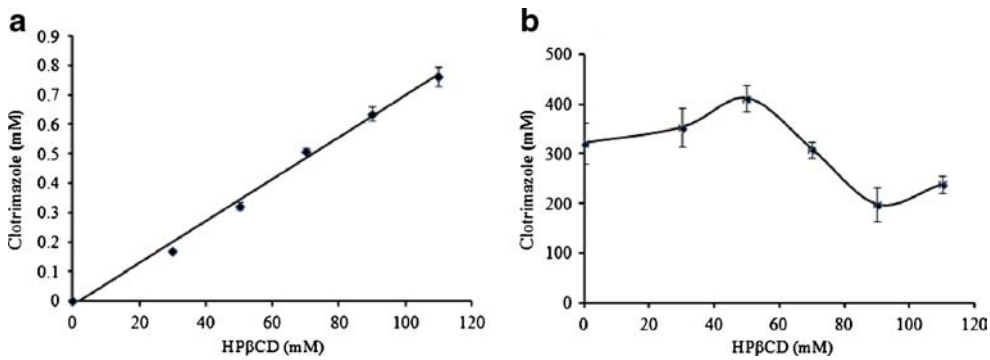

Fig. 1 Phase solubility diagram for CZ and CDs system at 37°C; (**a**) in aqueous solution, (**b**) in solvent system of EtOH:H_2O:BzOH with a volume ratio of 70:20:10.

Table II Solution Parameters before Electrospining of Spinning Solution Containing Various Ratios of Mixture Solvent and Different Amount of CZ. Each Value Represents the mean ± S.D. from Three Independent Experiments

Solvency ratio (EtOH:H_2O:BzOH)	Clotrimazole content	Viscosity (cP)	Conductivity (μS)	Surface tension (mN/m)
70:30:00	0%	216.80 ± 0.69	4.47 ± 0.75	28.39 ± 0.74
70:25:05	0%	224.40 ± 1.04	2.13 ± 0.42	28.47 ± 0.68
70:20:10	0%	228.13 ± 2.37	1.40 ± 0.26	29.40 ± 0.25
70:20:10	5%	216.33 ± 3.65	2.67 ± 0.15	29.34 ± 0.10
70:20:10	10%	219.80 ± 2.62	3.17 ± 0.31	29.12 ± 0.14
70:20:10	15%	225.83 ± 4.08	3.73 ± 0.32	29.68 ± 0.17
70:20:10	20%	230.47 ± 2.15	3.87 ± 0.12	29.39 ± 0.17

were 471, 663 and 665 nm, respectively. Nevertheless, the fibers became more uniform when BzOH content was increased to 10% because the solvent evaporated more slowly during the electrospinning process, and more CZ content could be dissolved. When the BzOH content was increased to 15%, the nanofibers could not be formed because the solvent did not evaporate. Therefore, an EtOH:H_2O:BzOH ratio of 70:20:10 was optimal for further experiment.

Additionally, electrospinning of PVP/HPβCD solutions containing various CZ content (containing 0, 5, 10, 15 and 20%wt CZ relative to the to polymer) were carried out from EtOH:H_2O:BzOH (70:20:10). By increasing the CZ content, the viscosity and conductivity of the solution were increased but these did not affect the diameter of the nanofiber mats (Fig. 3). The average diameters of nanofibers electrospun from solutions with 0, 5, 10, 15 and 20% wt CZ relative to the polymer were 665, 663, 667, 657 and 645 nm, respectively. Therefore, large amounts of CZ were incorporated into the nanofiber mats without affecting fiber diameter.

FT-IR Analysis

The FTIR spectra of the CZ powder, the blank nanofiber mats and 5–20% CZ-loaded nanofiber mats are displayed in Fig. 4a. The spectrum of the blank PVP/HPβCD nanofibers mats exhibited absorption peaks at 3,380.6, 2,941 and 1,093.1 cm^{-1} that were attributed to stretching vibrations of NH, OH, CH and CO stretching vibration respectively. The pure CZ powder exhibits dominant absorption peaks at 1,586.7, 1,490.7, and 1,304.9 cm^{-1} that correspond to the benzene ring stretches. The bands at 904.7, 823.68, and 744.59 cm^{-1} are assigned to the C–H stretches. The bands 1,081.4 cm^{-1} and 1,210.1 cm^{-1} correspond to the chlorobenzene and C–N stretching, respectively. The peaks observed in the CZ powder spectrum were also observed in spectra of the 5–20% CZ-loaded nanofiber mats. Therefore, CZ was incorporated into the nanofiber mats.

Differential Scanning Calorimetry (DSC)

DSC studies were undertaken to evaluate the physical state of CZ in the electrospun nanofiber mats, and the thermograms are displayed in Fig. 4b. The thermogram of the CZ powder exhibits an endothermic sharp peak at 145.13°C due to its melting temperature. A peak in the endothermic curve of the pure nanofiber mat (0% CZ) was also observed at 167.38°C. The melting points slightly increased to 173.59, 177.12, 178.12 and 186.49°C when the concentrations of CZ were 5, 10, 15 and 20%, respectively. The melting point of CZ is lower than the melting points of the PVP/HPβCD nanofiber mats, indicating that loading the CZ into the nanofiber mats does not affect their thermal behavior. Moreover, the absence of a detectable crystalline domain at a high CZ content, indicates that CZ was incorporated into PVP/HPβCD nanofiber mats in an amorphous state.

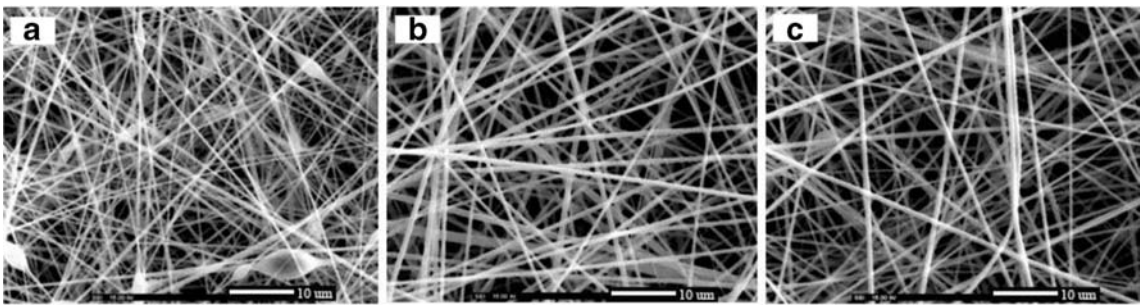

Fig. 2 The SEM image (1,000×) and diameter distribution of the PVP/HPβCD nanofiber electrospun from different solvent system ratio of EtOH:H2O:BzOH; (**a**) 70:30:0, (**b**) 70:25:5, (**c**) 70:20:10.

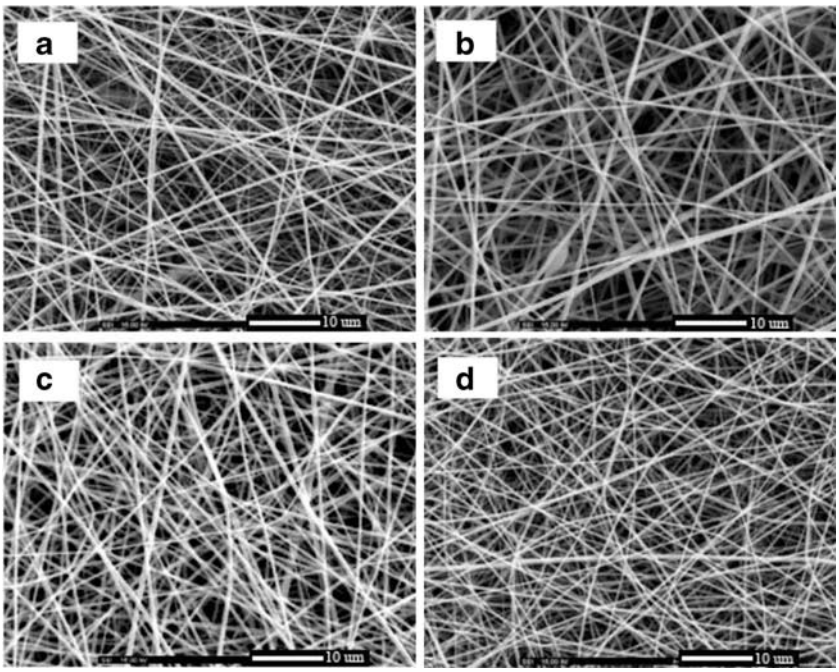

Fig. 3 The SEM image (1,000×) and diameter distribution of the PVP/HPβCD nanofiber mats electrospun from EtOH:H2O:BzOH solvent system ratio of 70:20:10 with a different CZ loading amount; (**a**) 5, (**b**) 10, (**c**) 15 and (**d**) 20%wt CZ to polymer.

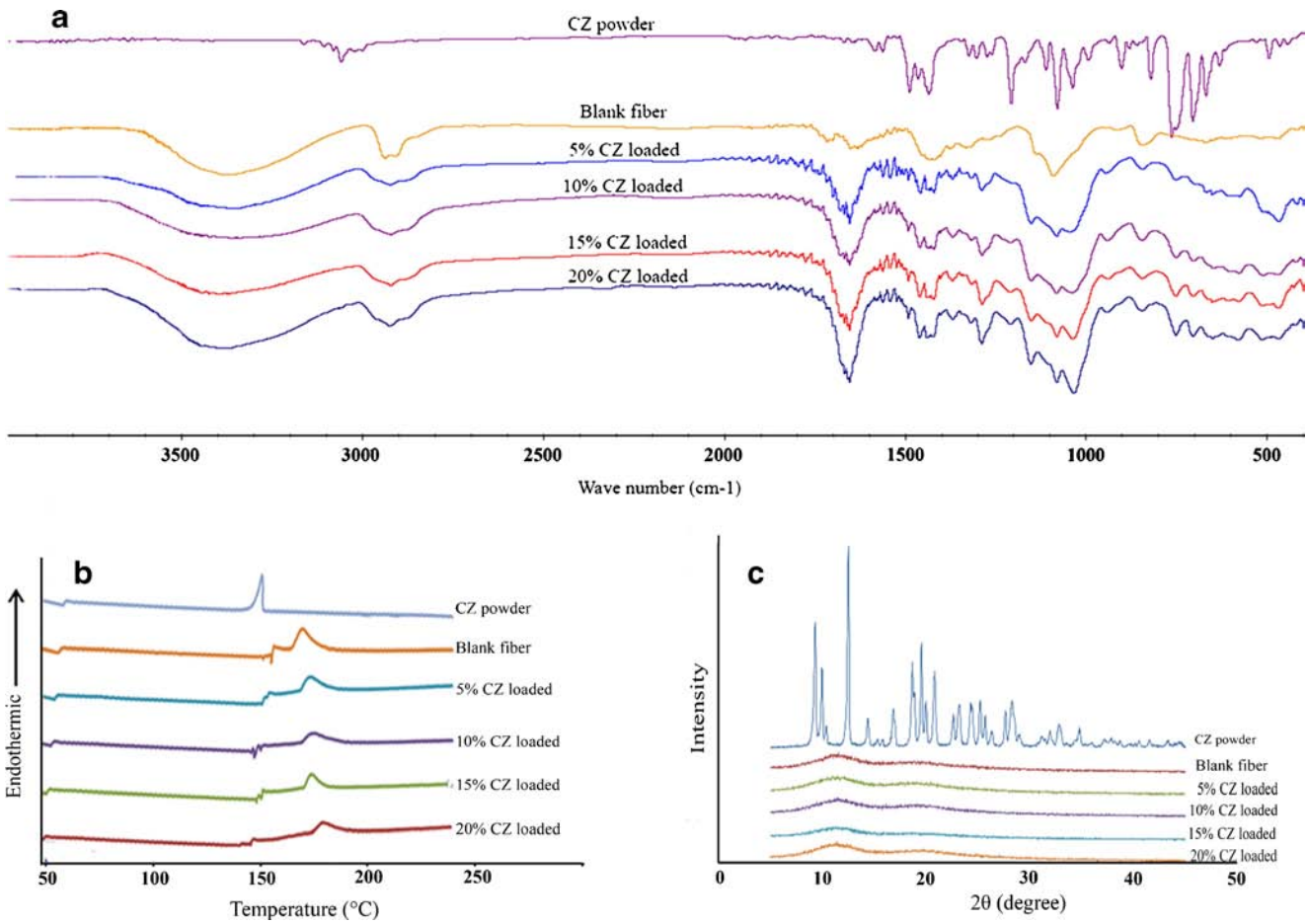

Fig. 4 (**a**) FT-IR spectra, (**b**) DSC thermogram and (**c**) X-ray diffraction pattern of the pure CZ powder and PVP/HPβCD nanofiber mats with different amount of CZ (0, 5, 10, 15, 20%wt CZ to polymer).

Table III The Entrapment Efficiency (%) and Loading Capacity (mg/mg) of CZ in PVP/HPβCD Nanofiber Mats. Each Value Represents the mean ± S.D. from Three Independent Experiments

Amount of CZ in nanofiber mats (% wt to polymer)	Entrapment efficiency (%)	Loading capacity (mg/mg)
5%	95.77 ± 1.43	0.046 ± 0.0007
10%	92.60 ± 2.52	0.084 ± 0.0022
15%	96.52 ± 5.26	0.126 ± 0.0069
20%	96.85 ± 4.51	0.161 ± 0.0075

X-ray Diffractometry (XPRD)

The powder X-ray diffraction patterns of the CZ powder, blank nanofiber mats and 5–20% CZ-loaded nanofiber mats are presented in Fig. 4c. The diffractogram of the CZ powder displayed strong crystalline peaks, indicating its high degree of crystallinity. However, no such peak was found in the diffractograms of the blank or 5–20% CZ-loaded nanofiber mats, indicating that CZ was incorporated into PVP/HPβCD nanofiber mats in an amorphous state.

Drug Content and Loading Capacity

Various amounts of CZ (0, 5, 10, 15, 20%wt to polymer) were incorporated in PVP/HPβCD nanofiber mats. The entrapment efficiency (% EE) and loading capacity for CZ in the PVP/HPβCD nanofiber mats are listed in Table III. The entrapment efficiency of CZ in the nanofiber mats was 92.60–96.85%, revealing efficient incorporation of CZ into the nanofiber mats. Increasing of the initial amount of CZ caused an overall increase in the amount of CZ

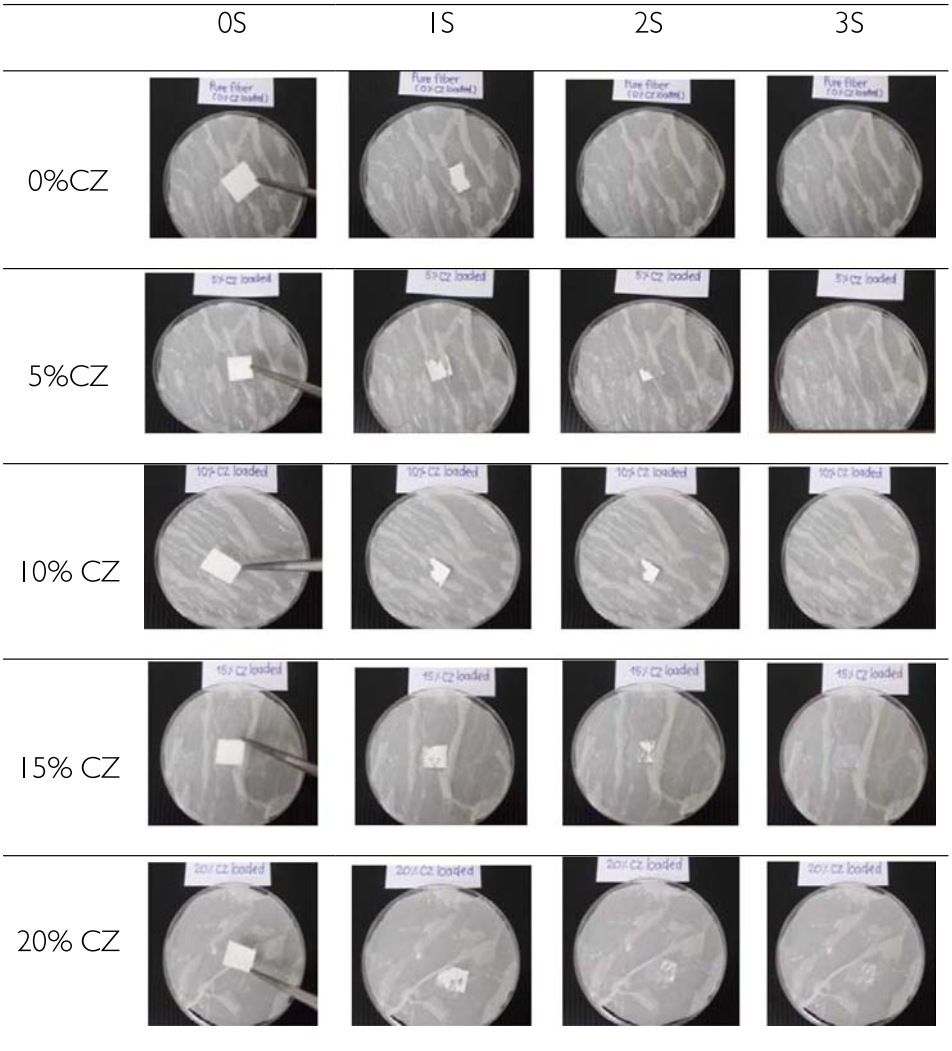

Table IV Wetting of the Nanofiber Mats Containing 0–20% CZ

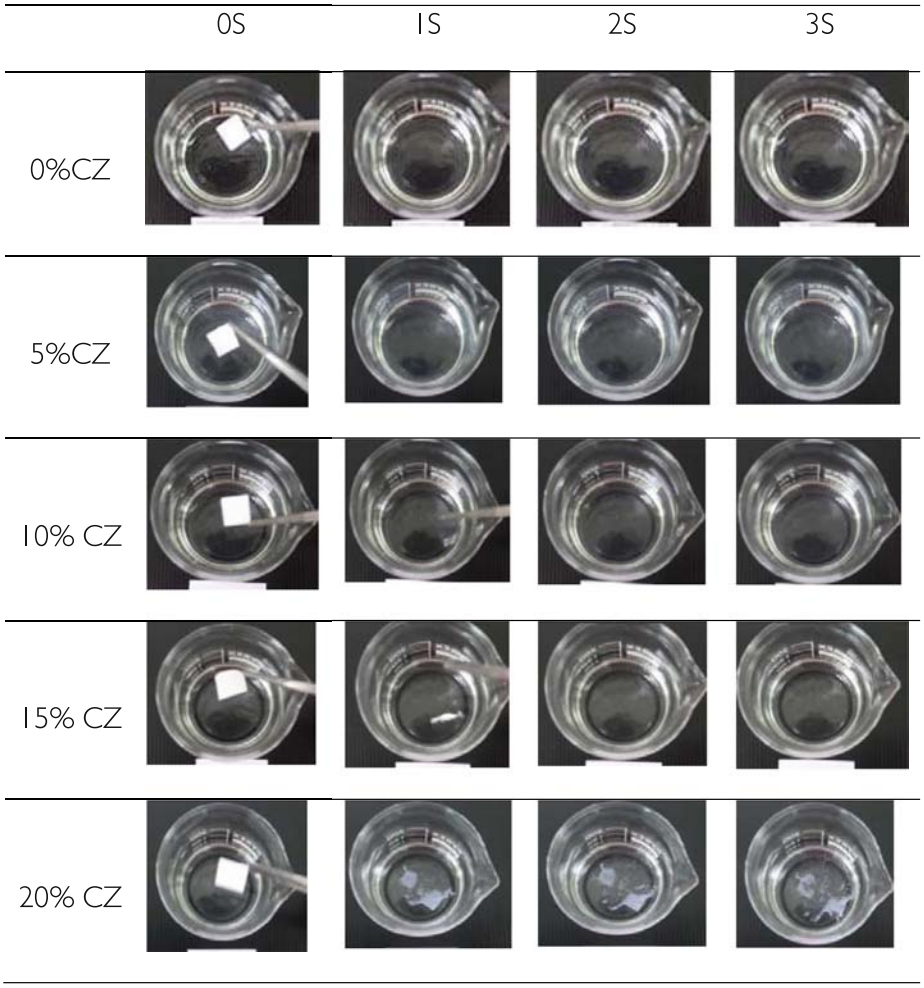

Table V Disintegration of the Nanofiber Mats Containing 0–20% CZ

entrapped in the nanofiber mats. The loading capacity for CZ in the nanofiber mats increased from 0.046 to 0.161 mg/mg of nanofiber when the initial amount of CZ increased from 5% to 20% wt to polymer.

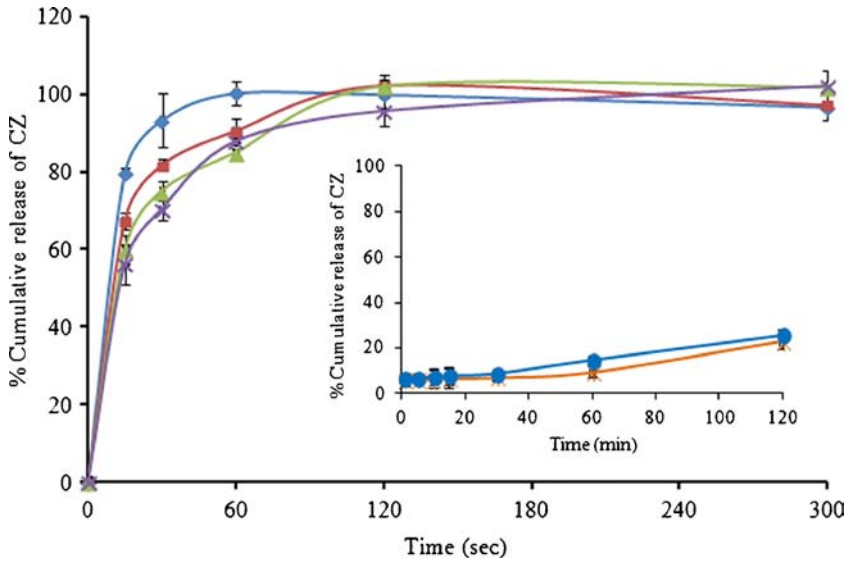

Fig. 5 Release profiles of CZ from CZ-loaded PVP/HPβCD nanofiber mats with different amounts of CZ: (♦) 5%, (■) 10%, (▲) 15% and (×) 20% to polymer. The insert represent release profiles of CZ from (●) CZ powder and (∗) CZ lozenges. The data are expressed as mean ± standard deviation from three independent experiments.

Wetting Time and Disintegration Time

The wetting time was investigated using wet tissue paper to mimic the moisture available moist environment in the mouth. The 0–20% CZ-loaded nanofiber mats were completely wetted, and lost their original white color within a matter of seconds after absorbing water from the wet paper as displayed in Table IV. The approximate wetting time of the nanofiber mats were 3 s. After placing the 0–20% CZ-loaded mats in artificial saliva, they quickly submerged, as illustrated in Table V. The mats lost their original white color, and finally disappeared. The disintegration or dissolution times of the mats containing 0–15% CZ were approximately 1 s. Therefore, electrospun nanofibers possessing highly porous structure allowed rapid penetration of saliva into the pores when placed in the oral cavity; thus, they showed rapid wetting and disintegration time which reflected the short time required to releasing the drug. However, the mats containing 20% CZ showed different disintegration behavior. They floated, lost their original white color, and then disintegrated into small pieces in the medium. This result revealed that high CZ content impeded the dissolution of the mat due to the lower solubility of CZ.

In Vitro Release

The release characteristics of CZ from the CZ-loaded PVP/HPβCD nanofiber mats with different CZ contents, CZ powder and CZ lozenges are shown in Fig. 5. CZ was rapidly released from the CZ-loaded electrospun nanofiber mats. After 1, 2, 2 and 5 min, CZ was completely released into the dissolution medium from 5, 10, 15 and 20% CZ-loaded nanofiber mats, respectively. On the other hand, only 23% and 26% of drug was released from lozenges and powder in 2 h,

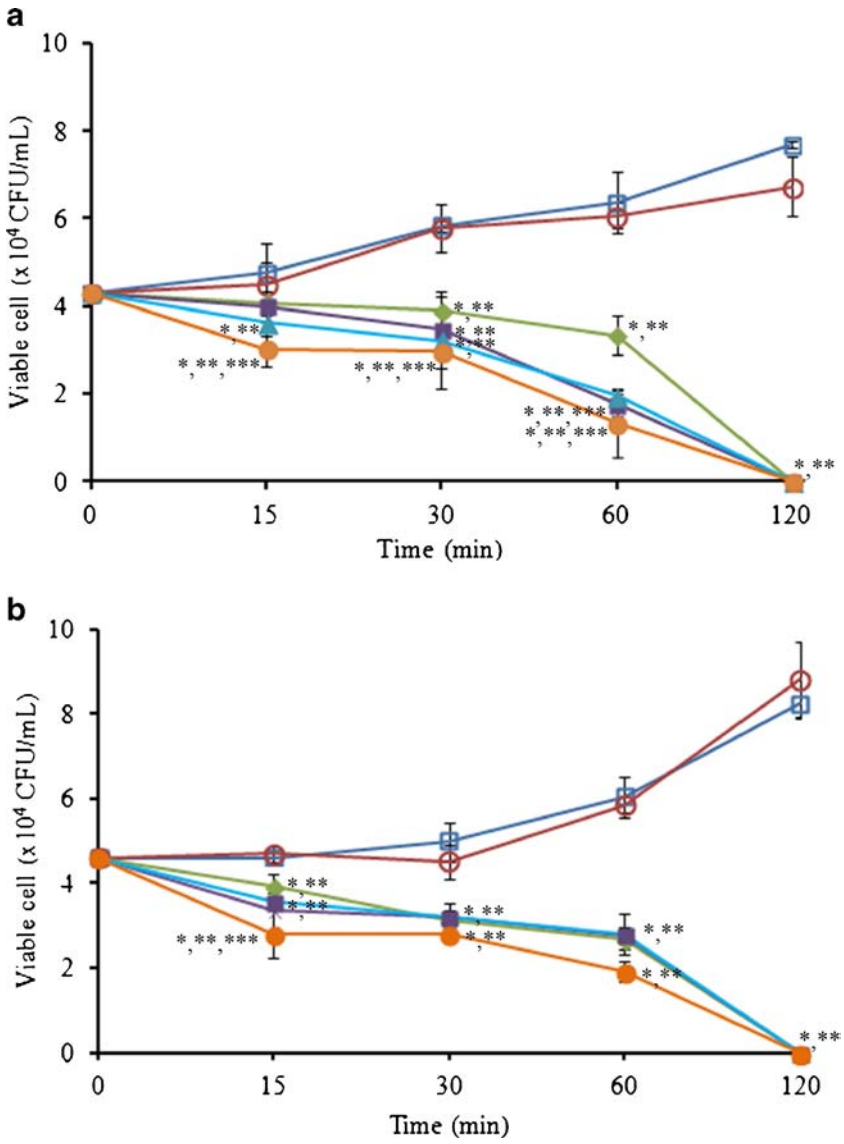

Fig. 6 Time kill plot of (**a**) *C. albicans* and (**b**) *C. dubliniensis* (CFU/ml) *versus* the treatment time for (□) the control and the 5 mg of CZ-loaded PVP/HPβCD nanofiber mats with different amounts of CZ: (○) 0%, (♦) 5%, (■) 10%, (▲) 15% and (●) 20% to polymer. The data are expressed as mean ± standard deviation from three independent experiments. * Statistically significant difference ($P < 0.05$) from control, **Statistically significant difference between from plain nanofiber mats (0% CZ), ***Statistically significant from 5% CZ loaded nanofiber mats.

respectively. The fast release of CZ from the nanofiber mats was caused by extremely high specific surface area and porosity of the mats, and PVP nanofibers could provide a fast-dissolving hydrophilic environment. Moreover, the electrospinning process left CZ in an amorphous state, facilitating drug dissolution in the medium. This characteristic was different from lozenges which required the time to disintegrate and subsequently dissolve and was different from CZ powder which was poorly soluble due to high lipophilicity and crystallinity.

Antifungal Study

The MIC and MFC values of CZ were obtained from susceptibility testing using a broth dilution assay. CZ was strongly active against *C. albicans* and *C. dubliniensis* with MIC values of 12.5 and 6.25 μg/ml and the MFC values of 25 and 12.5 μg/ml, respectively. These data agree with a previous report revealing that the MIC and MFC of CZ against *C. albicans* are 10 and 20 μg/ml (6).

The antifungal activity of the CZ-loaded nanofiber mats against *C. albicans* and *C. dubliniensis* was tested by counting the viable cells in a *Candida* suspension after making contact with the nanofiber mats with the suspension. Approximately 10^4 CFU/ml of the strain was exposed to nanofiber mats with different CZ loadings; furthermore, the CZ-loaded nanofiber mat with a final CZ concentration equivalent to 1 mg/ml in the *Candida* suspension was also tested in a time kill assay comparison with the CZ powder and the CZ lozenges at the same concentration of CZ. The CZ-loaded nanofibers could inhibit, killing it within 120 min of contact. In contrast, the

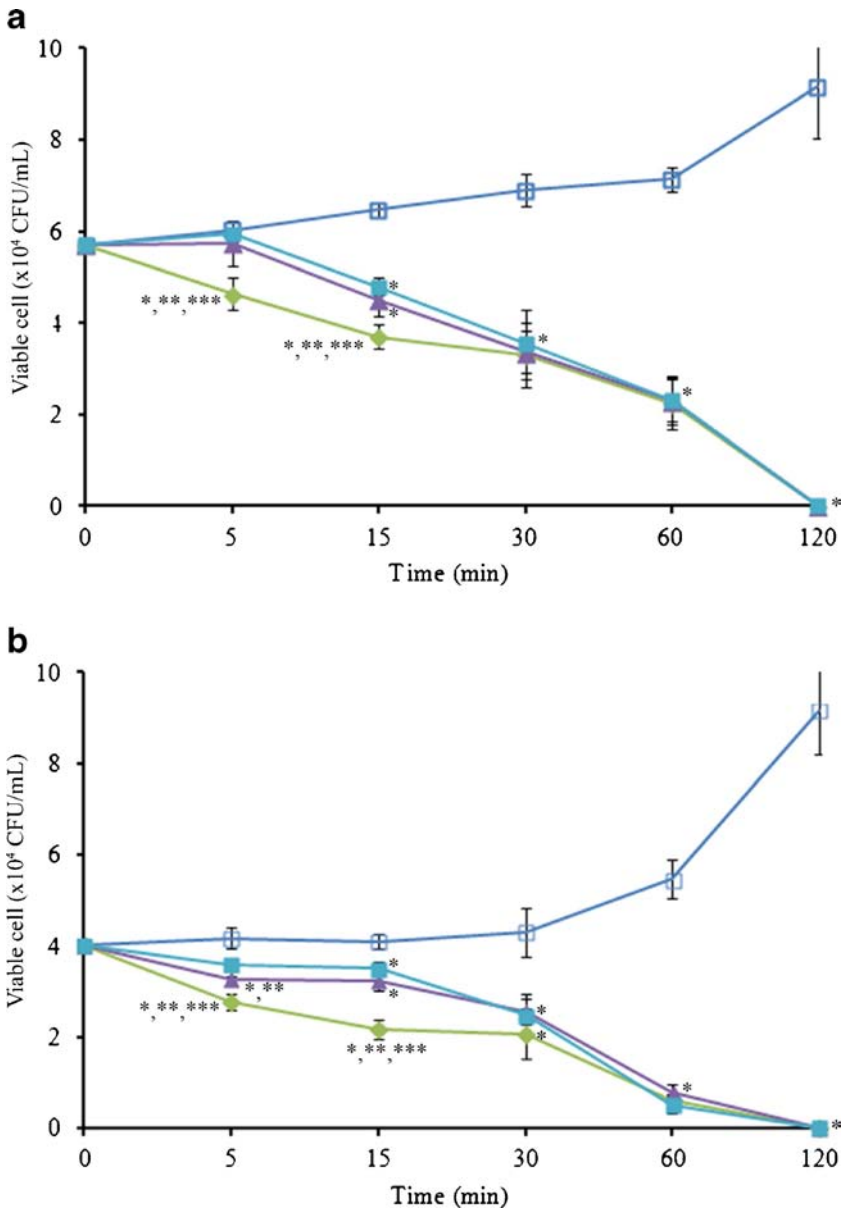

Fig. 7 Time kill plot of (**a**) *C. albicans* and (**b**) *C. dubliniensis* (CFU/ml) *versus* the treatment time: (□) the control, (♦) 20% CZ-loaded PVP/HPβCD nanofiber mats equivalent to CZ 1 mg/ml, (▲) CZ powder equivalent to CZ 1 mg/ml and (■) CZ lozenges equivalent to CZ 1 mg/ml. The data are expressed as mean ± standard deviation from three independent experiments. *Statistically significant ($P < 0.05$) from control, **Statistically significant between nanofiber mats and CZ lozenges and ***Statistically significant between nanofiber mats and CZ powder.

pure PVP/HPβCD nanofibers did not hinder the *Candida* growth. The killing abilities of the CZ-loaded nanofiber mats against *Candida* depended on the amount of CZ loaded as displayed in Fig. 6. The 20% CZ-loaded nanofiber mats significantly exhibited faster killing activity than 5% CZ-loaded nanofiber mats at 15, 30 and 60 min for *C. albicans* and at 15 min for *C. dubliniensis*. This result indicated the importance of CZ-loaded concentration on the killing rate. The higher the CZ-loaded concentration, the faster the killing rate was. In addition, the killing rate depended on the organism strains. This result was in accordance with the previous study of the metronidazole (MNA) electrospun poly(l-lactide-co-d/l-lactide) fibers that the antibacterial effectiveness of the aliquots from drug loaded

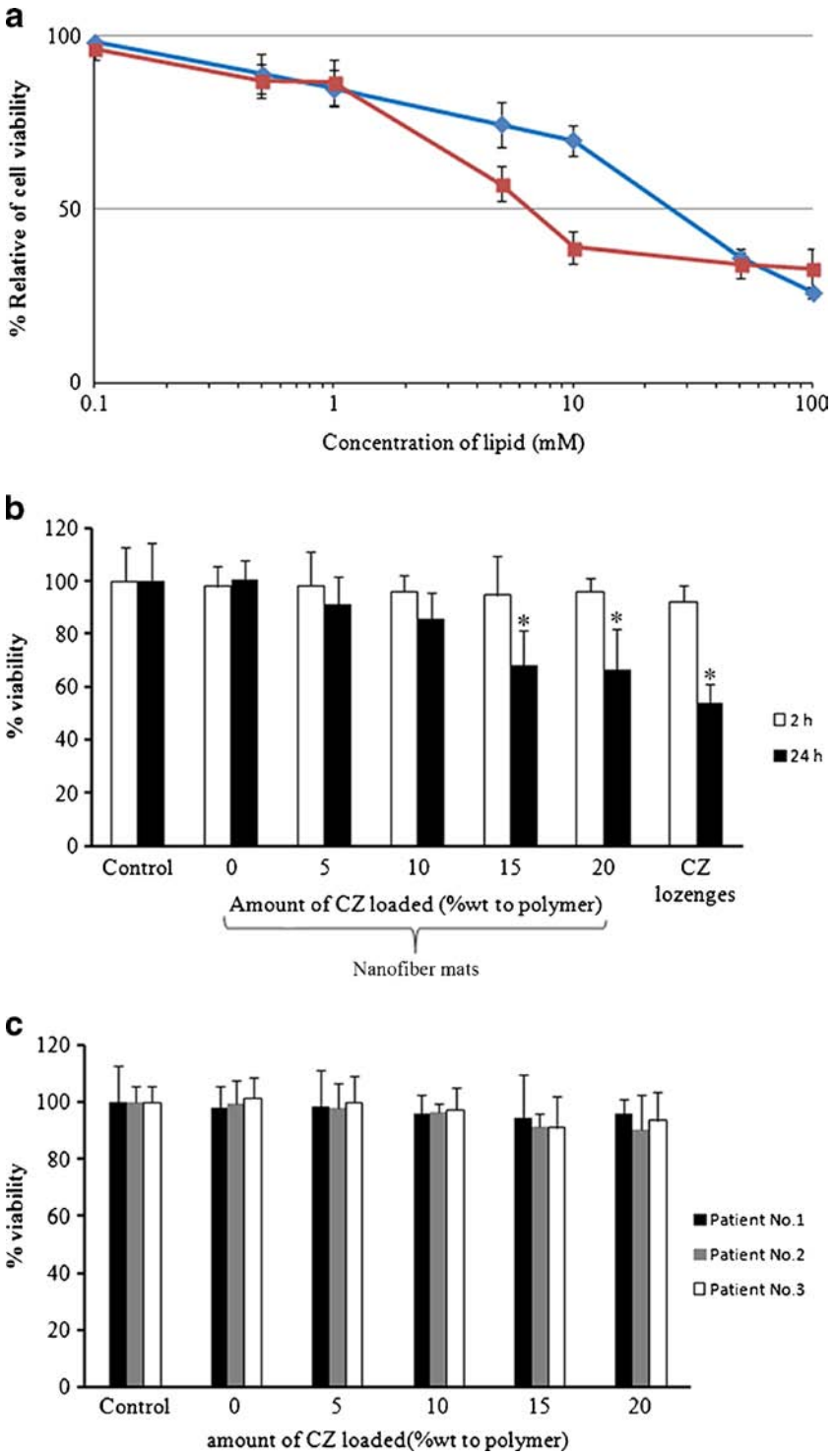

Fig. 8 The percentage cell viability in HGF cells of (**a**) CZ concentrations from 0.01 to 100 μg/ml and incubating for (♦) 2 h and (■) 24 h; (**b**) CZ loaded PVP/HPβCD nanofiber mats at different initial amount of CZ (0, 5, 10, 15 and 20%wt CZ to polymer) and CZ lozenges incubating for (□) 2 h and (■) 24 h; (**c**) CZ loaded PVP/HPβCD nanofiber mats at different initial amount of CZ in HGF cells derived from 3 patients: (■) patient #1, (▒) patient #2 and (▒) patient # 3. Each value represents the mean ± standard deviation of five wells. *Statistically significant ($P < 0.05$) from control group.

fibers containing different concentrations of MNA highly significant depended on bacteria species, concentration of MNA and incubation time (31). Relative to the CZ powder and CZ lozenges, the CZ-loaded nanofibers that had the same final CZ concentration significantly exhibited faster activity against both *C. albicans and C. dubliniensis* than CZ powder and the lozenges at 5 and 15 min for both *C. albicans and C. dubliniensis* (Fig. 7). This might be due to the rapid wetting/disintegration of the nanofibers and the amorphous state of the CZ; this amorphous state can dissolve more rapidly and easily than the crystalline state CZ powder or CZ lozenges; therefore, free CZ rapidly released from the mats caused faster antifungal activity. The CZ lozenges took longer time to disintegrate and dissolve; therefore, they displayed slower antifungal activity. The fast antifungal activity of the CZ-loaded PVP/HPβCD nanofiber mats makes them promising candidates for topical candidiasis applications (oral, vaginal, skin, etc.). For oral candidiasis treatment, not only the fast antifungal activity but also the sufficiently prolonged period where the drug concentration is above the minimum inhibitory concentration (MIC) would be more beneficial than one that only achieves a high CZ concentration for a short period. The suitable time period will be investigated *in vivo* CZ released from CZ formulation in healthy human volunteers (32).

Cytotoxicity Evaluation

The cytotoxicity of the CZ powder, CZ lozenges and CZ-loaded nanofiber mats was investigated by an MTT assay. Figure 8a shows the cell viabilities of CZ powder at CZ concentrations from 0.1 to 100 μg/ml and after incubation at pH 7.4 for 2 h and 24 h in the HGF cells. The results of the CZ powder showed a concentration-dependent cytotoxicity in HGF cells at pH 7.4 when incubated for 2 and 24 h as the IC_{50} were 24.9 and 6.1 μg/ml, respectively. The cytotoxicity effect of the extraction medium from CZ lozenges and 0–20% loaded nanofiber mats in HGF cells were evaluated and determine as % viability, as shown in Fig. 8b. For the CZ-loaded nanofiber mats, there was a significant decrease in the cell viability when the HGF cells were incubated with higher CZ loading in the nanofiber mats (15–20%) compared to the control ($p<0.05$) as illustrated in Fig. 8b. Cytotoxicity was observed only for a 24-h incubation with the extraction media from the nanofiber mats that contained high levels of CZ (15–20% wt. to polymer) (Fig. 8b). This result might be because the amount of CZ was very high and were toxic to fibroblast cells. However, the cytotoxicity of the cell viability was not statistically different in 0–10% CZ loading in the nanofiber mats. For the CZ lozenges extract medium which had the final CZ concentration equivalent to extraction medium of 20% CZ-loaded nanofiber mats, the cytotoxicity was not significant different from the extraction medium of 20% CZ-loaded nanofiber mats. Thus, the cytoxicity may be actually caused by CZ. After 2-h of incubation with extraction media from the nanofiber mats with different CZ amount and CZ lozenges, the cell viability remained similar to that of the control cells (cells not treated) at all concentrations. No significant difference in cytotoxicity was observed for CZ-loaded nanofiber mats in HGF cells derived from three different patients, as presented in Fig. 8c. The results indicated that CZ-loaded nanofiber mats were safe at the CZ 5–10% to polymer of the mats for 24 h and all formulations (5–20%) were safe for 2-h incubation.

CONCLUSION

PVP/HPβCD nanofiber mats containing clotrimazole (CZ) were successfully prepared via electrospinning using a EtOH:H_2O:BzOH solution with a 70:20:10 ratio (by volume) as a solvent system. Increasing the CZ content did not affect the diameter of the nanofiber s in the mats. CZ was rapidly released from the CZ-loaded electrospun nanofiber mats. The nanofiber mats exhibited antifungal activity more rapidly than the CZ powder or lozenges with low cytotoxicity. Therefore, these nanofiber mats may be promising candidates for oral candidiasis application.

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