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Development and *in vitro* evaluation of mucoadhesive gelatin films for the vaginal delivery of econazole

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Abstract

Several strategies have been explored to obtain effective econazole nitrate (ECN) concentrations at the site of application for a prolonged time. In this paper, different gelatin-based film formulations for vaginal application were investigated, containing ECN (10% w/w with respect to gelatin) as pure drug or as drug-solid dispersions (SD). For the production of SD, different polymers were evaluated: polyvinylpyrrolidone (PVP), Soluplus[®] (polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol graft copolymer) and Gelucire[®] 50/13 (mixture of mono-, di- and triglycerides of fatty acids, esters of PEG 1500 and free PEG). Gelucire[®]-SD showed the best solubility enhancement, increasing 9.2 times the ECN solubility in pH 4.5 solution respect to pure drug; DSC and XRD analysis confirmed the crystalline form of the drug. XRD results evidenced that all gelatin-based films, containing either the drug or the SD, underwent the topotactic transformation of ECN into crystalline econazole (EC), owing to a strong interaction between the drug and the gelatin. Films containing Gelucire[®]-based SD displayed lower brittleness and rigidity with respect to the other samples; moreover they demonstrated good structural integrity after 24 h of incubation in the acidic solution (swelling degree of about 350 %). Then, Gelucire[®]-SD based films were compared with the corresponding formulations cross-linked by genipin (2% w/w). The addition of genipin did not interfere with the drug-gelatin interaction. Gelucire[®]-SD based films showed similar release profiles to neat gelatin films, enhancing the drug release in the first 5 hours and controlling the EC release over time, avoiding the use of a crosslinking additive. Finally, gelatin films containing Gelucire[®] solid dispersion displayed good adhesiveness and anti-Candida activity. Overall, results support the potential use of this film formulation as noncytotoxic EC delivery system for the treatment of vaginal candidiasis.

Keywords

Econazole, solid dispersion, solubility, gelatin films, solvent casting, vaginal delivery, controlled release.

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1. Introduction

In the last decade, vaginal films have received a great deal of attention with respect to traditional vaginal dosage forms such as creams, gels and vaginal suppositories (Machado et al., 2013). Good retention ability, bio-adhesiveness, negligible discomfort, low cost, easy storage, decreased product leakage, ease of administration and wide formulation flexibility are the main interesting features of these thin strips (Valenta, 2005). Vaginal films have been formulated using a variety of polymers, both semi/synthetic and natural, such as polyacrylates, cellulose derivatives, polyvinyl alcohol, carrageenan, pectin and chitosan (Garg et al., 2005; Dobaria et al., 2009; Calvo et al., 2019b; Pereira Cautela et al., 2019). Recently, there has been a growing interest in the use of bioadhesive materials derived from natural origin, since these are generally recognized as safe, biodegradable and biocompatible in nature. Among them, gelatin, a polypeptide derived from collagen, is well recognized for its biocompatibility, low cost, plasticity and adhesiveness (Gómez-Guillén et al., 2011). However, its high solubility in aqueous solution has limited its application in vaginal film formulation design. In fact, the molecular weight, hydrophilicity and chemical structure of polymers dramatically influence the properties of the films such as mucoadhesion, disintegration time, drug release and mechanical strength (Machado et al., 2013).

To gain structural integrity at physiological conditions, gelatin needs to be stabilized. Physical treatments, as UV or γ irradiation or the most recent non-equilibrium atmospheric pressure plasma technology may be employed for the crosslinking of gelatin films (Dolci et al., 2018). Otherwise, crosslinking is ensured through polyion complexation, exploiting the different isoelectric point of gelatin: negatively charged acidic gelatin (gelatin A) or positively charged basic gelatin (gelatin B). Furthermore, chemical agents, as genipin, a natural product obtained from gardenia fruits (Bigi et al., 2002) may be added to form covalent bonds with the amino groups of gelatin.

Beside to the prolonged residence time at the site of infection, for effective local vaginal delivery of therapeutic agents, the dosage form needs to control the release of the active substance, while assuring its minimum effective concentration. Econazole nitrate (ECN) is an imidazole antifungal agent widely used for the treatment of mucosal candidiasis since its transmucosal absorption is negligible; thus, it could be useful for a topical therapy of vaginal candidiasis. However, the ECN poor water solubility along with the limited volume of the vaginal fluid (it was reported to be in the

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range of 2–3 g/24 h and it is decreasing with increasing age (Valenta, 2005) can compromise the drug availability (ElKasabgy, 2014, Firooz et al., 2015). Several strategies and different dosage forms have been explored to assure ECN concentrations significantly higher than its minimum inhibitory concentration (MIC) for a prolonged time. Polymer-lipid based mucoadhesive microparticles were designed as innovative vaginal delivery systems for ECN able to enhance the drug antifungal activity (Albertini et al., 2009). Another approach used to optimize the dissolution process of a different hydrophobic microbicide and thus its bioavailability, developed vaginal films as an amorphous solid dispersion of the drug in a hydroxypropylmethylcellulose and polyethylene glycol 400 mixture (Grammen et al., 2014). Other strategies to overcome the limitations of conventional therapy include nanoformulations. In particular, nanoparticles-in-film formulation have been considered for the vaginal administration of various microbicide drugs (das Neves and Sarmiento, 2017). More recently, a new ECN loaded chitosan-coated nanocapsule carrier, which might be used to load films, has been developed for the treatment of vaginal candidiasis (Calvo et al., 2019a).

The aim of this study was to develop an innovative vaginal mucoadhesive delivery system able on one hand to enhance the solubility of ECN, and thus its effective concentration at the site of action, and on the other to achieve a sustained drug release. Specifically, different ECN solid dispersions (SD) were prepared and characterized using different polymers (Soluplus[®], PVPs and Gelucire[®] 50/13) and loaded into gelatin-based films. Vaginal films were produced by solvent casting comprising either raw ECN or the corresponding SD. Cross-linked films by means of genipin (2% w/w) were also prepared for comparison purpose. Morphological and structural characterizations of the films were performed by means of scanning electron microscopy (SEM) and X-ray diffraction (XRD), and their mechanical, swelling and mucoadhesive properties as well as their *in vitro* drug release behavior were assessed. Finally, the antifungal activity of the developed films was evaluated *in vitro* against *Candida albicans* reference strain and clinical isolates recovered from human specimens. Effects of the films on cell viability and cell damage were assessed on a human genital epithelial cell line using two different assays.

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2. Materials and methods

2.1 Materials

Econazole nitrate (ECN) (1-[2-(4-chlorophenyl)methoxy]-2-(2,4-dichlorophenyl)ethyl) 1H-imidazole mononitrate; MW = 444.7 g/mol; Log P= 5.2; pKa 6.65) (Bachav et al., 2011) was supplied by Erregierre S.p.A. (BG, Italy). Type A gelatin (300 Bloom, Sigma Aldrich) from pig skin was used. Soluplus® (polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol graft copolymer: PCL-PVAc-PEG), Kollidon®30 (polyvinylpyrrolidone) and Kollidon®VA64 (polyvinylpyrrolidone-vinyl acetate) were kindly supplied by BASF (Ludwigshafen am Rhein, Germany). Gelucire® 53/10 (a mixture of mono-, di- and triglycerides, mainly mono- and diesters of palmitic (C16) and stearic (C18) acids), esters of PEG 1500 and also free PEG) was gently donated by Gattefossè (Milan, Italy). Genipin (Wako Chemicals, Japan) was used for crosslinking gelatin films. Mucin (from porcine stomach, Type III) was purchased from Sigma–Aldrich (Milan, Italy). Phosphate buffer (PB) solutions (pH 4.5 and pH 7.4), ethanol and double distilled water (DDW) were used throughout.

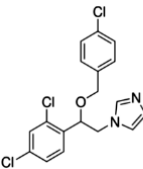
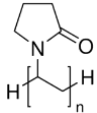
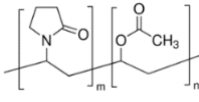
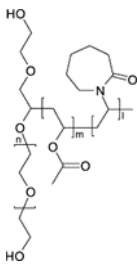
2.2 Preparation of Econazole Solid Dispersions

Solid dispersions containing Kollidon® (SDK) or Soluplus® (SDS) were prepared by solvent evaporation method: the powders were accurately mixed in a mortar and then solubilized in ethanol. The solutions were stirred for 30 minutes at room temperature (RT) and evaporated under low vacuum using a rotary evaporator (Buchi Rotavapor R-200, Flawil, Switzerland). Co-precipitate white crystals were obtained for SDS, while a slightly yellow rubbery solid was obtained for SDK. Gelucire® SD (SDG) was prepared by adding ECN to the molten Gelucire at 60°C. The suspension was stirred for 10 minutes then cooled down at -20°C for 10 minutes. All the obtained SD were pulverized in a mortar, stored at 4°C and characterized in terms of solubility and drug-carrier interactions. The labels and composition of each formulation are reported in Table 1.

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Table1. Composition of the solid dispersions: drug to polymer weight ratio (w/w)

SD	ECN	Polymers			
		Kollidon® 30 	Kollidon® VA64 	Soluplus® 	Gelucire® 50/13 <i>mixture of PEG-32 esters, free PEG and mono-, di- and triglycerides (composed mainly of stearic and palmitic acids)</i>
SDK	1	2	0.5	-	-
SDS	1	-	-	2	-
SDG	1	-	-	-	2

2.3 Solid dispersion characterization

2.3.1 ECN analysis by HPLC method

The HPLC method was previously described (Dolci et al. 2018) and conveniently modified. The HPLC system consisted of two mobile phase delivery pumps (LC-10ADvp, Shimadzu, Japan), an UV–vis detector (SPD-10Avp, Shimadzu, Japan) and an autosampler (SIL-20A, Shimadzu, Japan). The stationary phase was a Luna C18 column (150 mm x 4.60 mm x 5 µm; Phenomenex, Bologna, Italy). The mobile phase comprised of methanol and ammonium phosphate buffer 20 mM pH 2.5 (75:25 V/V). The flow rate was 1 mL/min and the detection wavelength was set at 230 nm. The retention time of ECN was about 5.4 min and the run time was set at 10 min. The injected volume was 20 µL. Quantitation was carried out using the linear calibration curve of ECN, obtained in the range of 0.5–40 µg/mL ($R^2 = 0.9999$). Specifically, six standard solutions were prepared by solubilizing the suitable amount of ECN in 10 mL of ethanol containing 150 µL of DMSO. Then the solutions were diluted with PB pH 4.5 to have the following concentrations: 0.5, 1, 5, 10, 20 and 40 µg/mL. A fresh ECN standard solution of 10 µg/mL was injected every day as control.

2.3.2 Solubility studies

An excess amount of ECN or of each SD was added to 10 mL of PB pH 4.5 under stirring at 37°C for 72 hours. Then the suspensions were centrifuged twice at 8000 RPM for 10 minutes and the

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supernatant analyzed by HPLC-UV/Vis. Measurements were performed in triplicate for each sample and the mean \pm S.D. was reported.

2.3.3 Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry measurements were performed using a PerkinElmer DSC 6 (PerkinElmer, Beaconsfield, UK). The instrument was calibrated with indium and lead for temperature, and with indium for the measurement of the enthalpy. The samples, weighing 10-12 mg, were placed into the DSC under a nitrogen flux (20 mL/min) and heated from 25°C to 250 C at a scanning rate of 10°C/min. The same procedure was used for the raw materials.

2.3.4 X-rays powder diffraction (XRD)

The powders were characterized by means of a PANalytical powder diffractometer equipped with a fast X'celerator detector. CuK α radiation was used (40 mA, 40 kV). The diffractograms were collected in reflection mode from 5° to 45°/2 θ with a step size of 0.033° and time/step of 1 s.

2.4 Film preparation

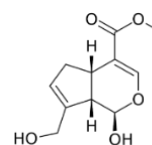
Gelatin films were prepared as described in our previous work (Dolci et al., 2018). Films containing 10% of ECN (w/w with respect to gelatin) were prepared by mixing the drug or the SD with gelatin in a mortar. The powders were suspended in DDW to obtain a 5% w/v gelatin concentration. The suspension was heated up at 50°C and stirred for 30 min. Then, 5 mL of each suspension were poured in 6 cm Petri dishes and allowed to dry at room temperature overnight. The obtained films were labeled GE10, GGE10, GSE10 and GKE10, as reported in Table 2. For the preparation of cross-linked films, genipin at 2% w/w (with respect to gelatin) was previously dissolved in 2 mL PB pH 7.4 and added to gelatin suspension. The mixture was stirred for about 20-30 minutes, during which the color of the solution turned from white / transparent to a pale blue. Then, 5 mL of each suspension were poured in 6 cm Petri dishes and allowed to dry at room temperature. After drying, films were rinsed with a Glycine solution (0.1 M in DDW) to remove the excess of genipin, repeatedly washed with DDW and air-dried at room temperature overnight. The produced films are labelled GE10gen and GGE10gen, as indicated in Table 2. As reference, unloaded gelatin films (G) and films containing the additives (GG, GS and GK) were prepared (Table 2). Dried films were removed from the Petri dishes, protected between two aluminum foils and stored into plastic bags at room temperature.

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Table 2. Gelatin (G)-based film composition.

Label	Theoretical ECN content (%w/w)**	SD	Genipin (% w/w)**
G*	-	-	-
GG*	-	-	-
GS*	-	-	-
GK*	-	-	-
GE10	10	--	--
GGE10	10	SDG	--
GSE10	10	SDS	--
GKE10	10	DSK	--
GE10gen	10	--	2
GGE10gen	10	SDG	2



*G, GG (G film containing Gelucire® 50/13), GS (G film containing Soluplus®) and GK (G film containing Kollidon®30 and Kollidon®VA64) represent the unloaded films containing the same amount of additives of the corresponding 10% drug loaded films.

**weighted with respect to gelatin.

2.5 Film characterization

Tensile tests

Stress-strain curves were recorded by using an INSTRON Testing Machine 4465 and the Series IX software package. Young's modulus (E), the stress at break (σ_b), and the strain at break (ϵ_b) were measured. Strip-shaped (3 × 30mm, thickness ranging from 0.086–0.100 mm) air-dried films, maintained at 24°C and at a relative humidity of 50%, were stretched at a crosshead speed of 5 mm/min. The thickness of the samples was determined using a hand-held digital micrometer (Mitutoyo, Japan) to an accuracy of 0.001 mm. At least ten samples were stretched for each composition.

Powder X-Ray Diffraction (XRD) analysis

The diffraction analysis of selected films was carried out in transmission mode by means of a PANalytical X'PERT PRO powder diffractometer using CuK α radiation (40 mA, 40 KV) and equipped

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with a PIXcell 1D detector. The 2θ range was from 4° to 35° with a step size of 0.067° and time/step of 10 s.

Infrared spectroscopy

ATR-FTIR analysis of the samples was carried out by means of an Agilent Cary 660 FTIR spectrometer equipped with an ATR sampling device, using a diamond crystal as internal reflection element. Infrared spectra were acquired at room temperature in absorbance mode, from 3900 to 400 cm^{-1} with a resolution of 2 cm^{-1} ; 32 scans were recorded for each spectrum.

Scanning Electron Microscope (SEM)

The samples were fixed on the sample holder with double-sided adhesive tape, sputter coated with Au under argon atmosphere by using a vacuum evaporator (Edwards, Crawley UK). Then films were examined by means of a scanning electron microscope (Philips XL-20) operating at 15 kV accelerating voltage.

Swelling measurement

Gelatin films were weighted in air-dried conditions and then immersed in 5 mL of PB pH 4.5 for different intervals of time up to 24 hours. Wet samples were wiped with filter paper to remove excess liquid and reweighted. The amount of adsorbed water was calculated as:

$$\text{swelling (\%)} = \frac{W_w - W_d}{W_d} \times 100$$

where W_w and W_d are the weights of the wet and the air-dried sample, respectively.

Determination of drug content

The ECN content within the film formulations was assayed by the HPLC method described in paragraph 2.3.1. Briefly, 50 mg of each film were dissolved in 10 mL of pure ethanol and stirred for 60 minutes; then 15 mL of acidic solution (composed of NaCl and HCl, pH 1.5) were added and the solution was stirred at 8000 rpm for 10 minute and assayed by HPLC.

Each formulation was analyzed at least in triplicate and the results are expressed as the mean of recovered drug (%) \pm SD.

In vitro release studies

The drug release from the films through cellulose acetate membranes (pore size: $0.45\text{ }\mu\text{m}$; thickness: $150\text{ }\mu\text{m}$) was performed using six modified Franz type diffusion cells. Films of 1 cm^2 (containing about 1.7 mg of drug) were allocated in donor compartment, while the receptor compartment

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phase was filled with 12 mL of PB pH 4.5 and maintained at 37°C by circulating water through the jacket of the lower compartment and constantly stirred at 100 rpm during the experiments. An aliquot of 0.8 mL was withdrawn at predefined time and analyzed by HPLC. The results are expressed as cumulative percentage of drug released and for each sample the mean of six replicates \pm SD was reported.

Mucoadhesive studies

The mucoadhesive properties were evaluated by using a microtensiometer opportunely modified as described in our previous work (Dolci et. al, 2018). Vaginal tissue, obtained from a local slaughterhouse (CLAI, Faenza, Italy), was immediately transported to the laboratory and used within 2 h. The tissue was suitably cut, washed and hydrated with PB pH 4.5 containing 1.5% (w/v) of mucin at 37°C for 15 min and then fixed with acrylic glue to the top of the homemade cell before starting the analyses. Each film (3 mm²) was fixed at the top plate using double-sided tape. Film and mucosa were put in contact for 30 seconds after which the top plate was moved up at a speed of 30 mm/min until their complete separation. The force was recorded on the display as a dyne/cm². This specific force was expressed as the force for cm² needed to detach the film from the mucosa. The results were then reported as mean values \pm S.D. and at least 10 replicate measurements were performed for each sample.

2.6 *Candida albicans* strains and in vitro antifungal susceptibility testing

The yeasts included in the present study were *Candida albicans* ATCC 10231 (American Type Culture Collection) and six isolates of *Candida albicans* recovered from urine specimens and genital swabs collected at the Microbiology Unit, St. Orsola Malpighi University Hospital, Bologna, Italy. The clinical strains were identified by standard procedures, including colony morphology on chromogenic agar (CHROMagar Candida medium, Becton Dickinson, Heidelberg, Germany) and confirmed by MALDI Biotyper System using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS, Bruker Daltonik, GmbH, Germany).

The effectiveness of the sample films to inhibit fungal growth was determined by means of disk diffusion assay performed on Sabouraud dextrose agar (SDA, Becton Dickinson, Heidelberg, Germany) plate. Briefly, the SDA surface was inoculated with a yeast suspension adjusted at 0.5 McFarland and prepared in sterile 0.9 % saline solution, and disk-shaped films (\varnothing = 6.0 mm) were

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laid down on the agar plate, perfectly adhering to the surface. As reference controls, sterile disks containing ECN 300 μg were included in each test. After 24 h at 37°C, the diameter of inhibition zone, corresponding to the fungal-free zone around the sample, was measured and expressed in millimeters. All experiments were performed in duplicate and in different days.

2.7 Cell viability and cell damage assays

The human cervix adenocarcinoma cell line HeLa (ATCC CCL-2) were used for *in vitro* cytotoxicity testing. Cells were routinely grown in Eagle's Minimal Essential Medium (E-MEM) supplemented with 10% fetal bovine serum (Carlo Erba Reagents, Milan, Italy), 100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin at 37°C with 5% CO_2 .

The safety profile of the film formulations was quantitatively evaluated by measuring cell viability and lactate dehydrogenase enzyme (LDH) release from damaged plasma membranes after treatment with some selected gelatin-based films. In detail, disk shaped samples were dissolved in 6 mL of culture medium for 24 h at 37°C, thereafter used for cellular experiments. The pH of the tested solutions was 6.8-7.0, therefore suitable for cellular investigations.

The cell viability was evaluated by using the CCK-8, Cell Counting Kit-8 (Dojindo Molecular Technologies, Rockville, MD, USA), that is a WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt]-based assay. WST-8 reagent is reduced by dehydrogenases in cells to formazan dye which is soluble in the tissue culture medium. The amount of the formazan dye generated by dehydrogenases in cells is directly proportional to the number of viable cells. The LDH released was determined by the Cytotoxicity LDH Assay Kit-WST (Dojindo Molecular Technologies). The measurement of the amount of released enzyme from cells is one of the major methods to assess the cell death.

For experiments, HeLa cells were seeded in a transparent 96-well plate at a density of 10^4 cells/well, grown for 24 h, and treated with 200 μL of solution in which the disks were dissolved. The following controls were included: untreated cells (control cells incubated with 200 μL of culture medium) and cells treated with the Lysis Buffer supplied by the LDH Assay kit (lysed cells). After 48 h, the culture medium was collected from each well, cell monolayer was washed with PB (pH 7.4), and 200 μL of fresh medium containing 20 μL of CCK-8 solution were added. After 2 h at 37°C, the absorbance was

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measured at 450/630 nm; results were expressed as the percentage of absorbance relative to the control cells.

In parallel, the collected cell-free supernatants were assayed for LDH release by adding a volume of the working reagent, and allowing the reaction to proceed for 30 min at room temperature in the dark. Thereafter, the stop solution was added, and the absorbance was measured at 490 nm. Data were expressed by the following equation:

$$\text{Cytotoxicity (\%)} = \frac{A-C}{B-C} \times 100$$

Where, A is the absorbance of the test solution, and B and C are the absorbances of lysed cells and control cells, respectively.

Experiments were carried out in triplicate, and in two independent assays.

2.8 Statistical analysis

A One-way analysis of variance (One-way ANOVA) was used to assess significant differences on the mechanical properties, mucoadhesion tests results and on anti-fungal properties for all considered samples. The significance was performed with Tukey's multiple comparison test. Differences were considered statistically significant with p values < 0.05.

3. Results and discussions

In the development of controlled release bioadhesive systems, both drug control release and swelling capability play an important key role. Films should also ensure appropriate concentration of the active ingredient at the site of application according to the treatment. Therefore, with the aim of improving the drug solubility and, consequently, its potential bioavailability, we first studied the effect of several polymers on the ECN solubility by designing three different solid dispersions, which were then loaded into gelatin films for vaginal application and compared to the neat drug-loaded films. In particular, the influence of different drug solid dispersions and of a cross-linker additive on properties of the gelatin matrix were investigated.

3.1 Characterization of the ECN solid dispersions

SD are widely used to improve the bioavailability of poorly water-soluble drugs (Tran et al., 2019). SD increase drug bioavailability owing to different mechanisms, which involve either the

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conversion of the crystalline drugs into amorphous forms or the formation of nano-crystalline phase using hydrophilic polymers (such as PVP and HPMC), or through the enhancement of drug wettability, dispersibility and supersaturated solubility by means of amphiphilic carriers (Bertoni et al., 2019a). In this study, polymers with different physical-chemical characteristics for solid dispersion production, as PVP, Soluplus[®], and Gelucire[®] 53/10 were tested for their ability to solubilize ECN. In particular, a blend of Kollidon[®] 30 and Kollidon[®] VA64, also known as copovidone, was selected as amorphous hydrophilic polymers able to form water soluble complexes and as amorphous APIs stabilizers (Lehmkemper et al., 2017; Lehmkemper et al., 2018), especially using PVPs blends (Forster et al., 2001). Soluplus[®] is an amorphous polymer with an amphiphilic chemical structure able to solubilize poorly soluble drugs in aqueous media (BASF, 2011; Carnon et al., 2013; Zi et al., 2019). Conversely, Gelucire[®] 50/13 is a semisolid waxy semi-crystalline material with amphiphilic nature used as solubilizer for poorly soluble compounds due to its property of forming micelles/self emulsifying systems (Albertini et al., 2015; Bertoni et al., 2019a, Bertoni et al., 2019b).

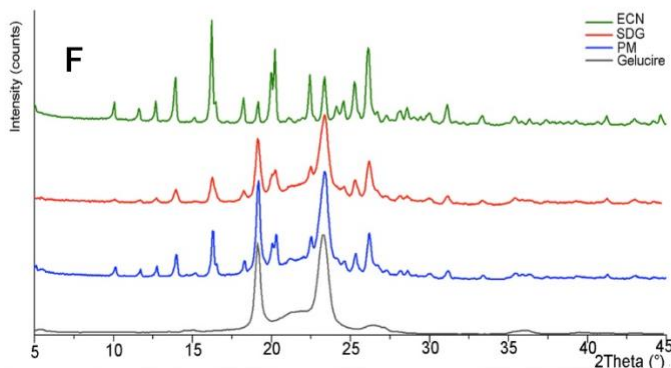
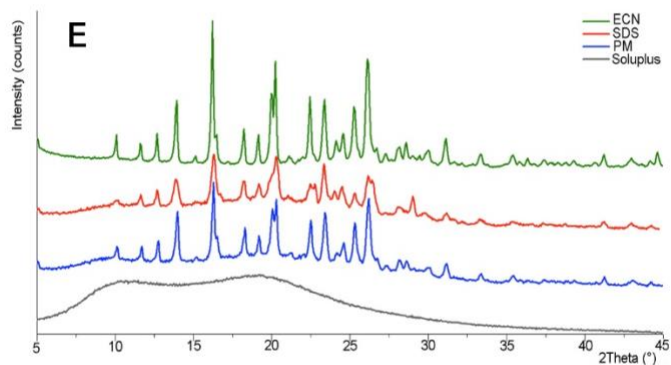
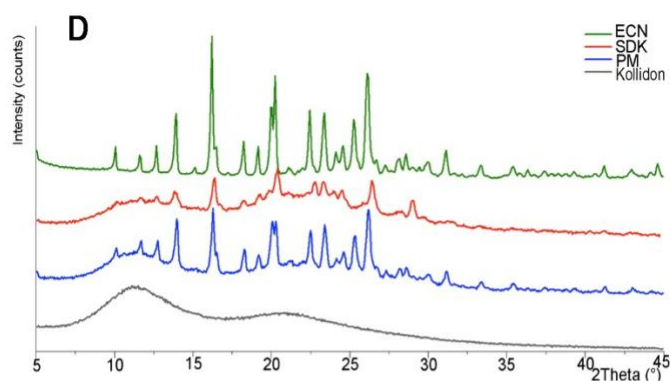
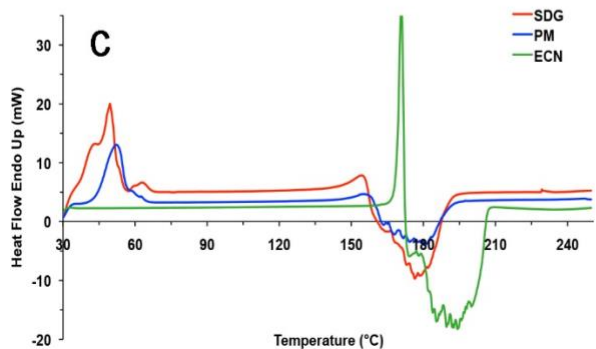
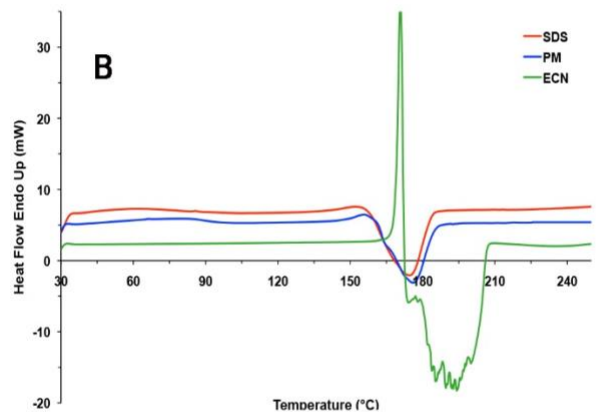
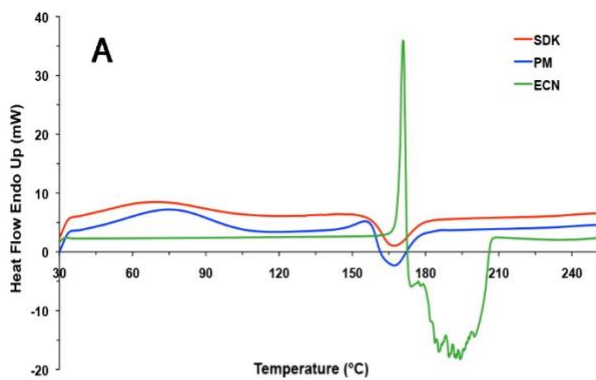
The solubility of pure ECN in PB pH 4.5 was $331 \pm 6 \mu\text{g/mL}$ *due to the acidic pH, which favored the solubilization of the weakly basic drug* (Table 1). All the SD increased the drug solubility. Specifically, Kollidon[®] and Soluplus[®] increased the ECN solubility 3.4 times ($1114 \pm 182 \mu\text{g/ml}$) and 2.5 times ($813 \pm 46 \mu\text{g/ml}$), respectively; while Gelucire[®] 50/13 increased the drug solubility of 9.2 fold ($3064 \pm 159 \mu\text{g/ml}$). Therefore, this additive demonstrated the higher ability in solubilizing ECN.

In order to detect possible drug solid state modifications during the SD preparation, characterization of the ECN-SD was carried out by means of DSC (Figure 1 A-C) and XRD (Figure 1 D-F). Figures 1A-C report the DSC curves of pure ECN, ECN-SD and of the corresponding physical mixtures (PM). Raw ECN showed an endothermic peak with a maximum at about 168 °C followed by a large and irregular exothermic peak, which made difficult the endotherm quantification (Albertini et al., 2009). Both PM and SD showed a lowering and broadening of the drug endothermic peak with respect to ECN. In particular, the melting peak of the drug shifted to about 156°C for all PM, reached slightly lower values for SDG (about 154°C, Fig. 1C) and was practically undetectable for SDS (Fig. 1B) and SDK (Fig. 1A). This modification may be due to the partial solubilization of the drug into the polymer during the DSC scan (hence justifying the similar behavior of the PM). In fact, ECN may partially solubilize either in the molten Gelucire at about 60°C (Albertini et al., 2009), or in

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the polymeric carriers, since their T_g values are lower than the melting point of ECN (T_g = 70°C, 149°C and 101°C for Soluplus, Kollidon 30 and VA64, respectively) (BASF, 2011; Haaf and Straub, 1985). On the other hand, the lowering and broadening of the ECN endothermic peak could be related to the presence of a drug nano/micro crystalline phase within the SD, as previously reported in literature (Hasa et al., 2013).



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Figure 1: A-C) DSC curves of ECN-SD (red line) pure ECN (green line), and of the corresponding physical mixtures (PM, blue line); D-F) XRD patterns of additives (black line), physical mixtures (PM, blue line) and of ECN-SD (red line) compared to that of pure ECN (green line).

In order to better define the solid state of the ECN within the SD, samples were analyzed by X-rays diffraction. Figures 1D-F report the XRD patterns of additives and of SD compared to that of pure ECN and of physical mixtures (PM). The diffraction patterns of SDS (Fig. 1 E) and SDG (Fig. 1F) samples displayed the permanence of the ECN crystalline structure; the reduced intensity of the reflections may be simply due to drug-additive blend (1:2 weight ratio). Regarding SDK (Fig. 1D), a certain reduction of the drug crystallinity could be appreciated, since the majority of the reflections decreased in intensity and those at about 26 and 32° of 2θ completely disappeared. Thus, despite the partial drug amorphisation using PVPs, the SD with Gelucire 50/13 showed the highest enhancement in drug solubility, while maintaining the drug crystallinity. These results suggest that the solubility improvement was not related to the drug solid state in the SD, but rather to the ability of the amphiphilic carrier to increase API solubility as a result of the formation of a micellar dispersion (Shaker, 2018; Bertoni et al., 2020).

3.2 Characterization of vaginal films containing the SDs

Gelatin-based films, containing either the drug or the SD, were then prepared at 10% w/w ECN. In a recently published paper, it was found that ECN was able to interact with gelatin by base-acid reaction between the basic ε-amino groups of gelatin residues and the acidic hydrogen of ECN, resulting in transformation of econazole nitrate into econazole, EC (Dolci et al., 2018). Since this topotactic transformation occurred during film preparation, films containing the SD were characterized to evaluate the eventual drug modification, even in the presence of polymers. Figure 2 reports XRD patterns (Figure 2): all the reflections matched with those of EC powder (orange line), thus confirming that the presence of additives did not prevent the base-acid interaction between ECN and gelatin.

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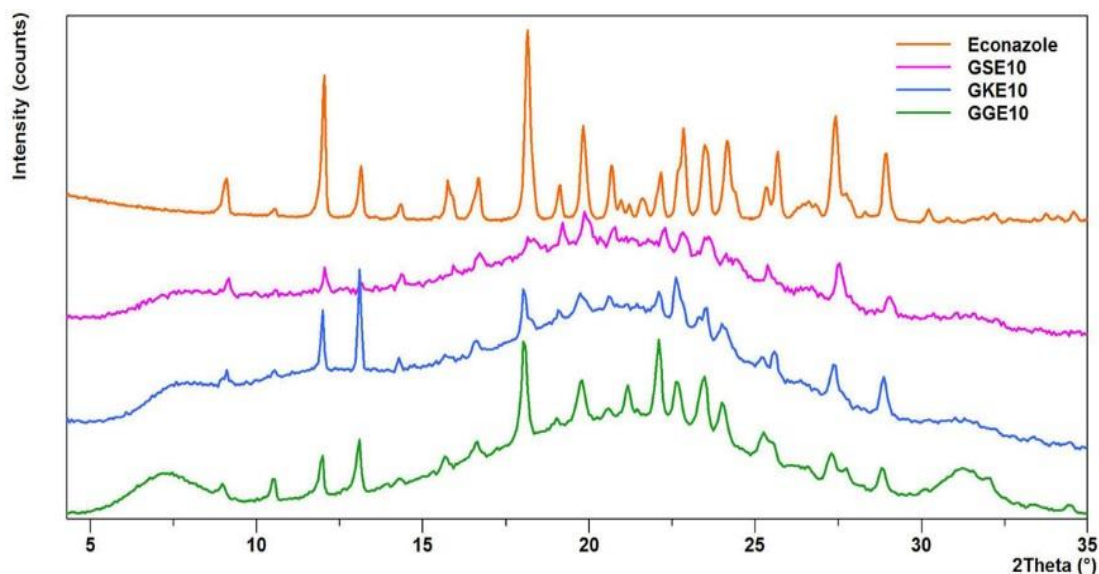


Figure 2: XRD analysis of EC powders (orange line) and of gelatin-based films: GGE10 (green line), GKE10 (blue line) and GSE10 (pink line).

Films should also display adequate mechanical properties, thus tensile properties of films were evaluated. The values of stress at break (σ_b), deformation at break (ϵ_b) and Young's Modulus (E) are reported in Table 3.

G films are brittle and rigid, with high elastic modulus and stress at break. Addition of ECN (compare G and GE) did not significantly affect mechanical parameters as previously reported (Dolci et al., 2018), while the introduction of the additives induced a significant decrease of the stress at break and of the elastic modulus for all the tested formulations. Films GG, GK and GS showed a noticeable decrease of all the mechanical parameters with respect to G. When SD were added, the most remarkable differences between GKE10 and GSE10 lie in stress values, which are almost double with respect to GK and GS, whereas GGE10 displayed the lowest stress and modulus and the highest deformation. These features highlighted that GGE films were the less brittle and rigid materials among all the tested compositions.

Table 3. Thickness, stress at break, σ_b ; strain at break, ϵ_b ; and Young's modulus, E; of gelatin-based films. Each value is the mean of ten determinations and is reported with its standard deviation.

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Films	Thickness (μm)	σ_b (Mpa) \pm SD	ϵ_b (%) \pm SD	E (Mpa) \pm SD
G	91	93 \pm 6	7 \pm 2	3600 \pm 200
GG	115	40 \pm 5	5 \pm 2	1630 \pm 250
GK	105	37 \pm 8	2.0 \pm 0.4	2500 \pm 300
GS	101	30 \pm 7	3.0 \pm 0.3	2090 \pm 160
GE10	110	83 \pm 7*	8 \pm 2*	3640 \pm 250*
GGE10	115	56 \pm 6	11 \pm 2	2070 \pm 140
GKE10	107	67 \pm 5	7 \pm 2	2300 \pm 200
GSE10	102	73 \pm 7	7 \pm 2	2700 \pm 240
GE10gen	98	78 \pm 10*	6 \pm 1*	3400 \pm 200*
GGE10gen	100	62 \pm 10	4 \pm 1	2640 \pm 240

*data from Dolci et al., 2018 here reported for comparison purpose.

Gelatin films were highly soluble in aqueous media swelling of about 1000% after few hours in saline solutions (Amadori et al., 2015). The incorporation of ECN increased the gelatin solubility, as GE10 films were not recoverable in pH 4.5 PB after 30 min (Figure 3A). When SD of different compositions were added, all the films swelled considerably lower than G and GE10: in particular, GKE10 and GGE10 showed a swelling degree of about 450 and 350 %, respectively, after 5 hours. GSE10 displayed an intermediate swelling degree. At longer times all the films gradually dissolved: after 24 hours only GGE10 and GSE10 films were still well recognizable (Figure 3B). Therefore, comparing the gelatin films, those containing Gelucire[®] 50/13 demonstrated good flexibility and structural integrity after 24 h of incubation in aqueous media. Based on these results, only gelatin films containing SDG were further investigated and cross-linked with genipin.

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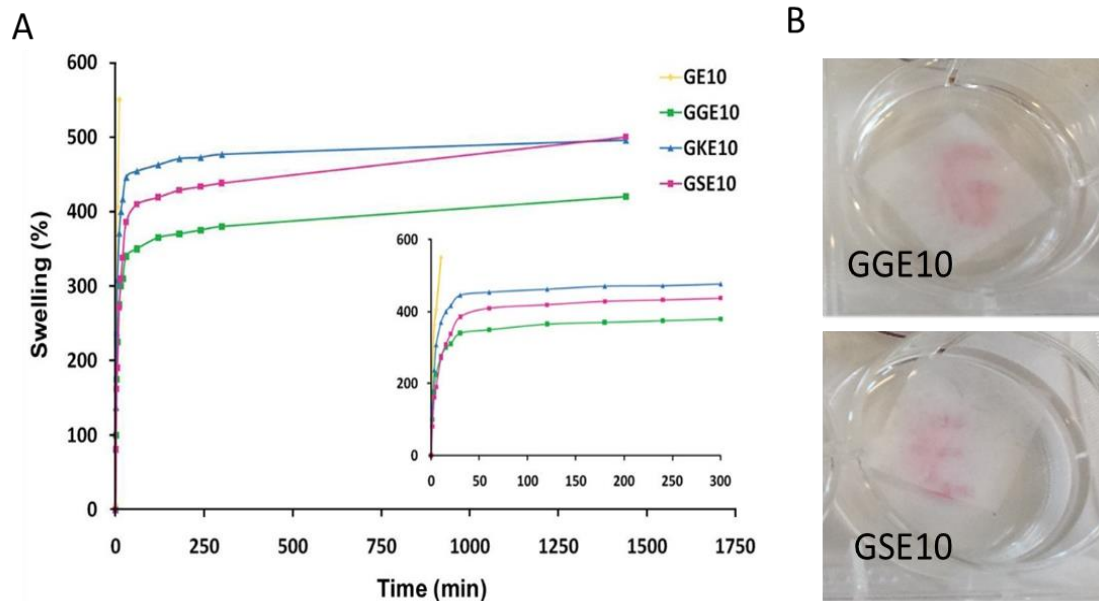


Figure 3: A) Influence of the additives on the swelling ability of G-films; B) Images of GGE10 and GSE10 films after 24 hours in pH 4.5 PB.

3.3 Characterization of gelatin films containing SDG

Gelatin-based films containing ECN at 10% w/w, added either as a powder or as a SD, were then studied. Due to the high solubility of the gelatin films, stabilization with a suitable crosslinking method is mandatory for mucosal application. Genipin, a natural product obtained from gardenia fruits, is a valid alternative to the cytotoxic glutaraldehyde useful for cross-linking gelatin (Bigi et al., 2002; Dolci et al., 2018). Therefore, gelatin films crosslinked with genipin (2% w/w) were investigated and compared with the uncross-linked ones regarding mechanical properties, morphology, drug solid state and drug-excipient interactions, swelling behavior and drug release.

The elongation at break (ϵ), the stress at break (σ_b) and the Young's modulus of the films were measured from the stress-strain curves. The results (Table 3) evidenced that genipin crosslinking did not modify the tensile properties of the films (compare GE10/GE10gen), while the addition of SDG provoked a significant decrease of both stress at break and elastic modulus.

XRD patterns of genipin cross-linked films (GE10gen) revealed the permanence of EC crystals (Dolci et al., 2018). The XRD analysis of the films containing SDG (GGE10) showed that the treatment with genipin did not alter the EC solid state with respect to the uncross-linked samples (Figure 4).

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These results suggested that neither Gelucire[®] 50/13, nor genipin interfered in gelatin-ECN acid-base interaction.

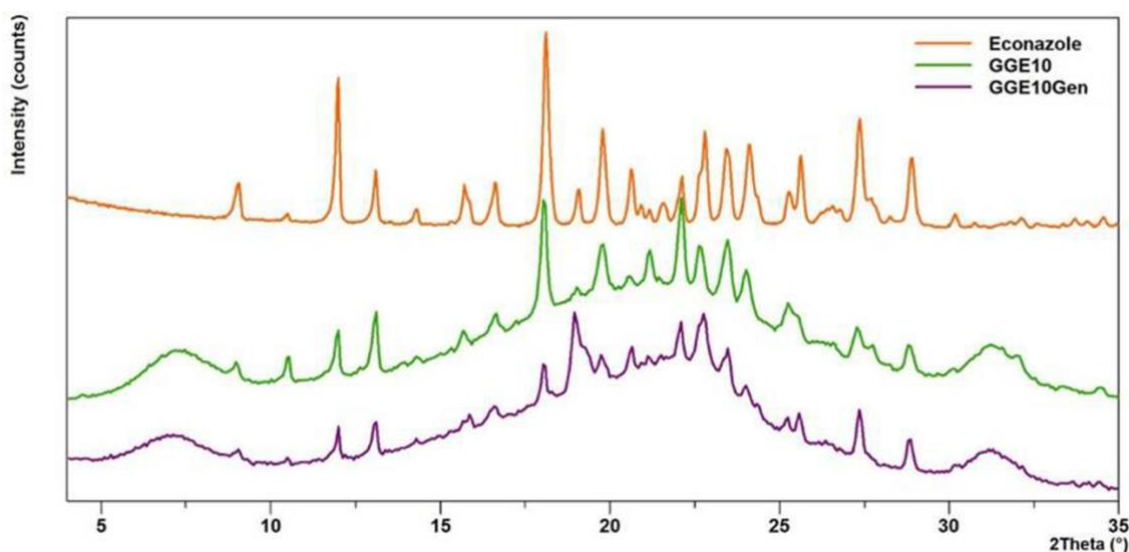


Figure 4: XRD patterns of EC, GGE10 and GGE10gen.

The IR spectrum of the drug (not reported) evidenced several characteristic peaks at 3174 and 3108 cm^{-1} (aromatic C–H stretching vibrations); 1585 and 1547 cm^{-1} (aromatic C–C and C–N stretching vibrations); 1218 cm^{-1} (aromatic C–Cl stretching vibrations); 1108 cm^{-1} (C–O–C stretching vibrations); 828 and 803 cm^{-1} (out of plane C–H deformation vibration) and finally at 761 cm^{-1} (out of plane C–H deformation vibration of the tri-substituted phenyl group) (Albertini et al., 2009). FT-IR spectra recorded on GE10 showed several absorption bands corresponding to amide I, II and III typical of gelatin superimposed on those of EC (Kujawski et al., 2016) and after genipin crosslinking (GGE10gen) no remarkable differences of peaks shapes and intensities were evidenced, as previously observed in Dolci et al., 2018. The IR spectrum of Gelucire[®] 50/13 (not reported) displayed a broad band between 3100–3600 cm^{-1} (stretching of free OH groups), at 1738.5 cm^{-1} (stretching C=O group), at 1469.5 cm^{-1} (C–H deformation of alkyl group), at 1113.7 cm^{-1} (–C–O stretching) and at 963.3 cm^{-1} (double band, characteristic of the polyethylene glycol groups) (Bertoni et al., 2019a). Comparing the FT-IR spectrum of GE10 to the GGE10 ones (Figure 5), the film containing SDG showed all the characteristic adsorption bands of both gelatin and Gelucire, suggesting the absence of interactions between the components of the formulation.

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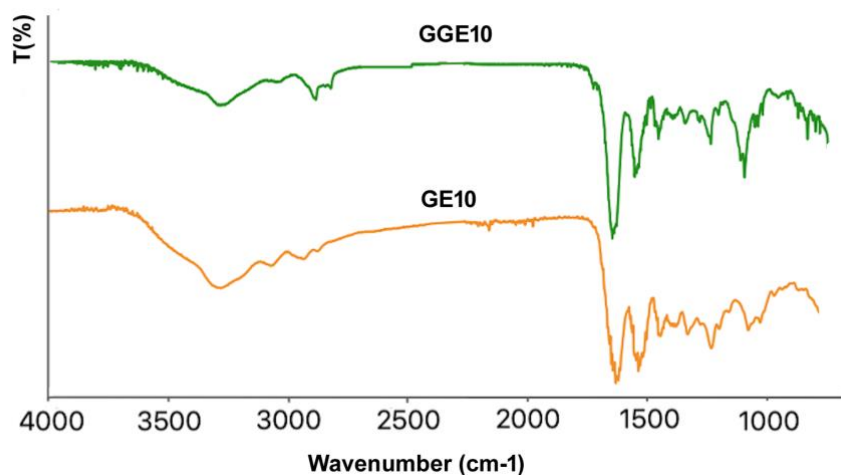
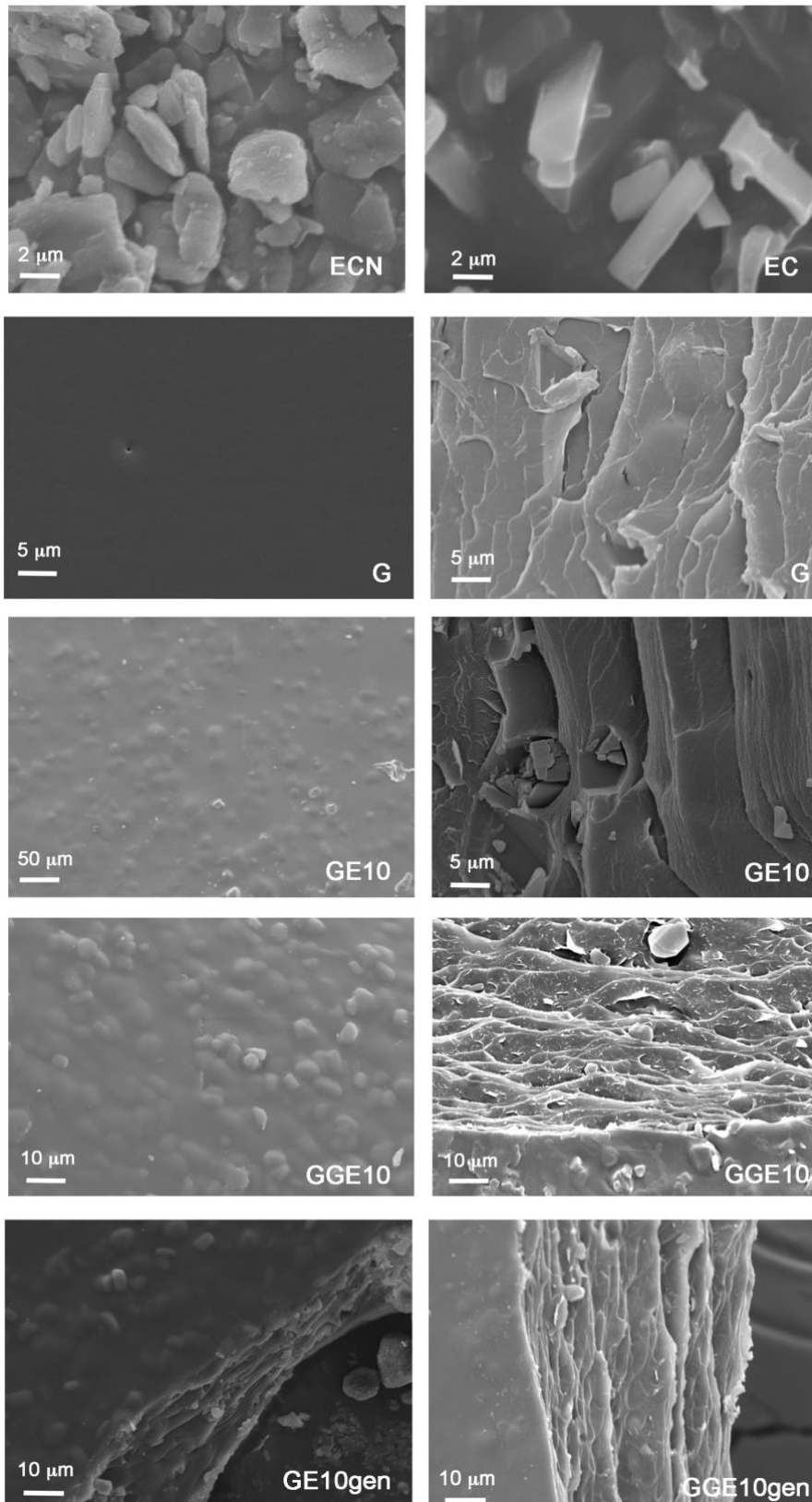


Figure 5: FT-IR absorption spectra collected on GE10 and GGE10.

SEM images, reported in Figure 6, showed the difference between the drug crystals of pure drug (ECN) and the modified drug within the film formulation (EC). The unloaded film (G) presented a smooth surface. Films loaded with the drug or with the SDG (GE10 and GGE10) presented small crystals of the drug underneath the film surface due to the suspended drug within the polymeric matrix, as previously observed by Tejada et al., 2018. The images of GE10 and GGE10 revealed a uniform distribution of the drug into the film with the EC crystals distinctly recognizable. Moreover, SEM analysis evidenced that there are no differences in terms of drug crystal morphology in the presence or absence of the solid dispersion. The morphology of the cross sections showed the typical leaflet structure of gelatin film, even if GGE10 films showed a more compact structure than GE10 ones. Moreover, the morphology did not change after chemical cross-linking (GE10gen and GGE10gen).

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Figure 6: SEM images of ECN crystals, EC crystals, G film (surface, left and cross-section, right), GE10 film (surface, left and cross-section, right), GGE10 film (surface, left and cross-section, right), and images of GE10gen and GGE10gen.

The HPLC analysis of the samples evidenced that the drug recovery was in the range 86-93% (92.6 ± 0.5 , 89.6 ± 1.2 , 87.9 ± 1.8 and 89.8 ± 1.9 for GE10, GGE10, GE10gen and GGE10gen, respectively), suggesting that a certain amount of drug remained bounded with gelatin after its topotactic transformation, thus decreasing the free drug extent, regardless the presence of the additives (neither Gelucire[®] 50/13, nor genipin). To confirm this hypothesis, uncross-linked films (GE10 and GGE10) were also prepared by aprotic solubilizing ECN or SDG and gelatin in DDW at pH 1.5 while maintaining the same proportion and conditions of stirring and temperature previously reported for film preparation. The pH was adjusted at 1.5 by adding HCl (6M) before the solvent casting process. The results confirmed a greater availability of free drug, as the drug recovery was about the 100% for all the samples (100.2 ± 2.1 and 101.2 ± 2.1 for GE10 and GGE10, respectively), confirming our hypothesis.

The release profiles of both uncross-linked films and genipin cross-linked films are reported in Figure 7A. The drug release profile from GE10 and GGE10 was controlled during the first 5 hours and reached about 40% of drug dissolved (Fig. 7A): GGE10 films displayed a greater burst release than neat GE10 films due to the high solubility of the SDG. In particular, the amount of drug released after 2 hours was 21,5% and 12% for GGE10 and GE10, respectively. Then, both films exhibited a sustained slow drug release up to 24 hours. In particular, the amount of EC released was 70% and 55% for GE10 and GGE10, respectively. Noteworthy, GGE10 film appeared swelled but still intact (Fig.7B) as previously observed during swelling studies), while GE10 dissolved forming a gel layer. GE10gen e GGE10gen showed a more controlled drug release up to 24 hours (Fig 7A) and intact swollen films were still present at the donor sites of Franz cells at the end of the experiment.

These results indicated that all film formulations controlled the drug release up to 24 hours and only the uncrossed-linked ones (GE10 and GGE10) could achieve a higher drug concentration (about 40%) at the site of application during the first 5 hours. Comparing the release profiles of GE10 and GGE10 after the first 5 hours, the addition of Gelucire[®] 50/13 enabled a more controlled drug release, due to its amphiphilic nature with respect to the hydrophilic gelatin. Then, genipin cross-

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linking exhibited a greater effect on the release profile of films without Gelucire: after 5 hours the difference of drug released from GE10 and GE10gen is about 21%, while it was merely 13 % from GGE10 and GGE10gen, suggesting that Gelucire might have reduced the extent of cross-linking between the ϵ -amino groups of gelatin and genipin. After 24 hours, around 55% and 62% of drug were released from GGE10 and GE10gen films, respectively. Therefore, Gelucire[®] 50/13, due to its peculiar amphiphilic property, ensured an appropriate release of the active ingredient, both enhancing the drug release at the beginning of the treatment and controlling the drug release over time, as genipin. Moreover, genipin is relatively expensive and imbues crosslinked-gelatin films with a dark blue color, which could represent a limit for some applications.

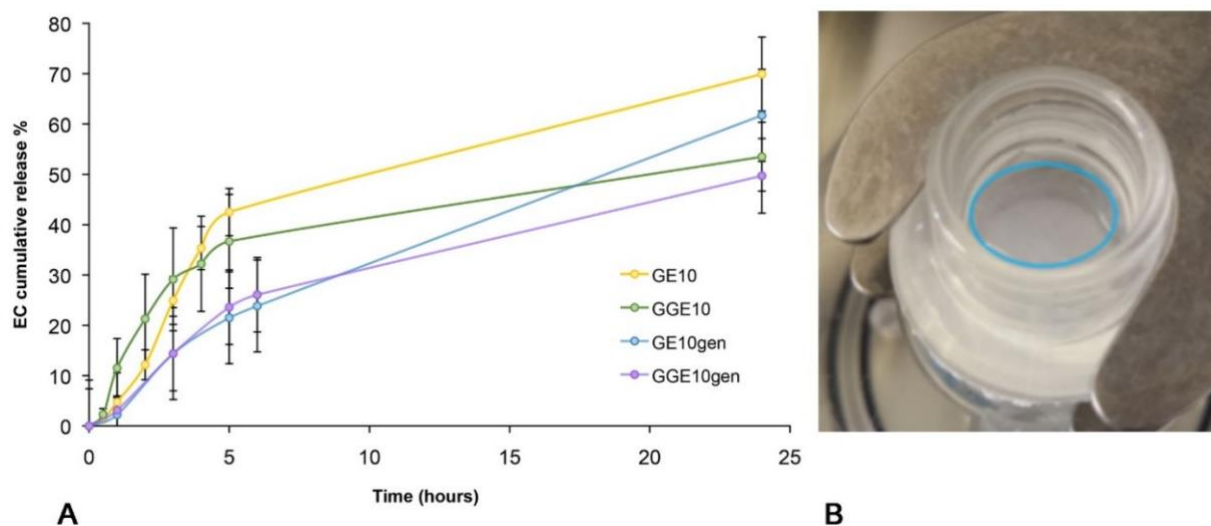


Figure 7: Release studies performed using Franz diffusion cells. A) EC release profiles from both uncross-linked gelatin-based films and genipin cross-linked gelatin-based films; B) Swelled GGE10 film after 24 hours in the donor chamber of the Franz cell system.

Finally, the ability of the film to adhere to the mucosa, measured as the force required to detach the film from the mucosa, was investigated and the results are reported in Figure 8. GE10 film did not present a significant mucoadhesive strength, owing to the high water solubility of gelatin in the absence of crosslinking. On the contrary, the addition of genipin to the ECN loaded film (GE10gen) showed a strong increase of mucoadhesive properties. It is interesting to note that the mucoadhesive strength value of GGE10 resulted to be comparable to the cross-linked GE10gen film

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($p > 0.05$). Therefore, the addition of Gelucire[®] increases the structural integrity of the gelatin film allowing the adhesion of the film to the mucosa pivotal for gelatin film application, while avoiding the cross-linking step. Furthermore, a small difference was observed between the samples GGE10 and GGE10gen (** $p < 0.01$). Therefore, these results highlight that the presence of Gelucire[®] solid dispersion within gelatin films had a significant effect on the strength of adhesion to the mucosa tissue, thus improving the residence time at the site of application.

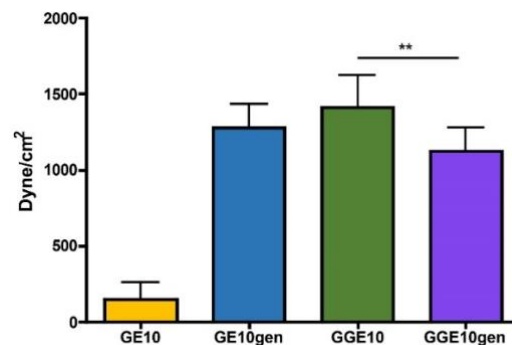


Figure 8: Mucoadhesive strength values of the films: GE10, GE10gen, GGE10 e GGE10gen (** $p < 0.01$)

3.4 Biological activity

The anti-*Candida* activity of the different gelatin-based films was assessed *in vitro* against both *C. albicans* reference strain ATCC 10231 and six clinical isolates recovered from genitourinary tract infections. The diameters of the inhibition zones are reported in Figure 9. Data indicated that the gelatin films inhibited fungal growth with the same effectiveness, and no statistically significant differences were measured in the diameter values between these formulations and the ECN positive control (ECN 300 μg). Considering the ECN amount loaded on the film samples, and the weight of gelatin-based films, the drug concentration ranged from 300 to 340 μg , depending on the formulation. The four gelatin-based films displayed similar inhibitory activity towards the reference and the clinical strains, which are representative of pathogens circulating in the population, however the best result was achieved for GGE10. The cross-linking step with genipin slightly decreased the effectiveness of the films as diameters of the fungal-clear zones reduced, even not at

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significant levels. Overall these data confirm the suitability of Gelucire® as polymer for ECN delivery system.

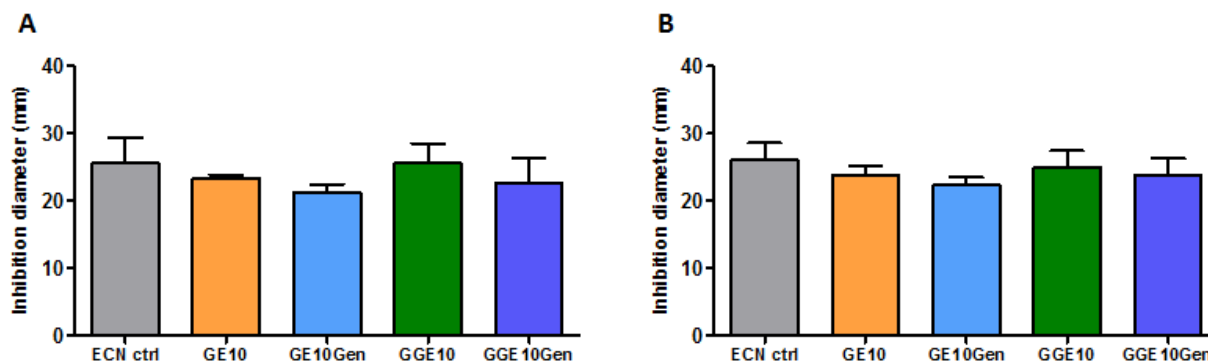
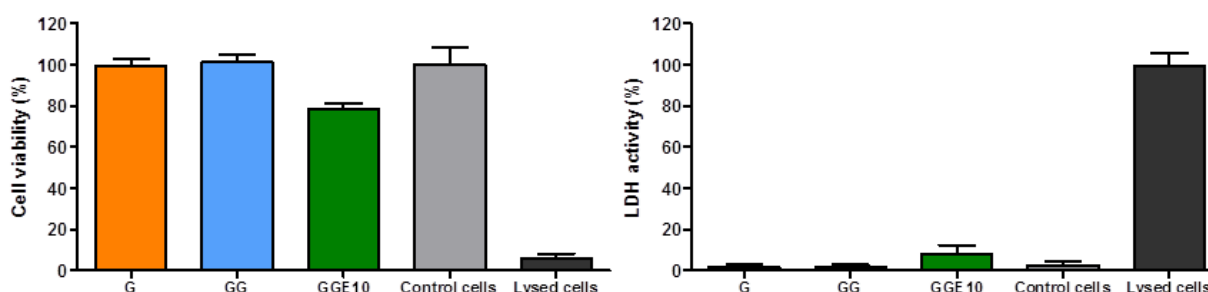


Figure 9: Anti-*Candida* activity of gelatin-based films loaded with ECN against the *C. albicans* reference strain (A), and the six clinical isolates (B), whose results were pooled together. Data are expressed as the diameter (in mm) of the clear free-zone measured around the disk shaped samples. Negative films (GG10 and Ggen) were included in the analysis and no inhibition was measured.

As an *in vitro* proof-of-concept on the safety of the gelatin-based films, G, GG, and GGE10 disk-shaped samples were dissolved in cell culture medium and solutions were used to treat HeLa cells. To obtain an accurate result in cytotoxicity assay, the samples were evaluated by different methods that are the determination of NADH in living cells and the release of LDH from dead cells. Results at 48 h of culture are reported in Figure 10. As a sample is considered cytotoxic when its viability is less than 70% in comparison to untreated cells (control cells), none of the tested solutions is cytotoxic, at the used experimental conditions. Only a slight reduction of cell viability (22%) was measured for GGE10, while analysis of cell damage by LDH evaluation confirmed the safety of this sample as LDH activity was measured at negligible level (<10%).



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Figure 10. Cell viability assay (A) and LDH release (B) of HeLa cells following 48 h of culture with the experimental samples. Results are percentage values relative to both control cells and lysed cells.

4. Conclusions

Econazole-loaded vaginal films based on gelatin alone and films containing a drug solid dispersion based on different polymers (Soluplus[®], PVPs and Gelucire[®] 50/13) were successfully prepared and characterized. The solubility of ECN increased for all SD, though the drug remained almost crystalline within the SD. Once the SD was combined with gelatin matrix, XRD patterns demonstrated the occurrence of the base-acid interaction between the drug and the polymer, resulting in transformation of ECN into EC. Among the tested formulations, GGE films demonstrated good flexibility and swelling ability combined with a certain structural integrity after 24 h of incubation in PB pH 4.5. Additionally, GGE films displayed greater adhesiveness and activity against *Candida albicans* than gelatin films alone and those crosslinked with genipin, without a cytotoxic effect. Thus, the use of Gelucire[®] 50/13 within the polymeric film matrix could represent a valid alternative to the use of chemical crosslinkers to obtain gelatin-based delivery systems for prolonged drug release. In conclusion, the overall *in vitro* results demonstrated that formulated films containing Gelucire improved the drug solubility, while controlling the drug release for an extended period of time, suggesting their potential use for the treatment of vaginal candidiasis with a single application per day. Moreover, being films very flexible in dose according to the final size, they enable a customized dose according to the need.

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