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3D printed clotrimazole intravaginal ring for the treatment of recurrent vaginal candidiasis

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ABSTRACT

Vulvovaginal candidiasis is a vaginal infection caused by the fungal pathogen *Candida albicans* that, most commonly, affects women of reproductive age. Its first-line treatment consists in topical applications of conventional drug formulations (*e.g.*, creams, gels, tablets) containing imidazole drugs. The treatment involves single or multiple daily applications and, in the case of recurrences, daily administration of oral antifungal drugs for up to one month.

Intravaginal rings are flexible, biocompatible medical devices that, compared to conventional drug formulations, offer the possibility of a controlled vaginal drug delivery over a determined period with a single application, thus increasing patient compliance. Among innovative manufacturing techniques, in recent years, fused deposition modeling 3D printing has emerged in the pharmaceutical field to produce different therapeutics combining drugs and polymers. This technique allows to print objects layer by layer with many different thermoplastic materials after a computer-assisted design.

Thermoplastic polyurethanes are flexible and biocompatible materials that can be efficiently employed for the manufacturing of drug release systems, already utilized to prepare vaginal devices. In this work, we produced a clotrimazole-loaded intravaginal ring by fused deposition modeling 3D printing combining the drug with thermoplastic polyurethane using hot melt extrusion. The rings were computer-designed and then printed with two different drug concentrations (*i.e.*, 2% and 10 % w/w). The intravaginal rings were first tested in an agar-diffusion test to evaluate their effectiveness against *C. albicans*; and the 10% loaded ring was selected for further studies. Drug release was evaluated in two different media (*i.e.*, 50% ethanol and vaginal fluid simulant) showing a sustained release over a period of seven days. Next, *in vitro* time-kill analysis against *C. albicans* in simulated vaginal fluid was performed and displayed a complete growth inhibition after 5 days, compared to the control.

These results suggest a potential application of these 3D printed intravaginal rings for the treatment of vulvovaginal candidiasis and for the long-time treatment of recurrences.

Keywords: Topical application; vaginal device; additive manufacturing; controlled release.

1. INTRODUCTION

Vulvovaginal candidiasis (VVC) is among the most common vaginal infections in women of reproductive age caused by the opportunistic fungal pathogens *Candida albicans* and occasionally by other *Candida sp.* or yeasts (Gonçalves et al., 2016). These fungi are known to asymptomatically colonize many areas of the body of healthy individuals as part of the resident microbiota, including the gastrointestinal and genitourinary tracts. However, frequent antibiotic therapies, contraceptive use, alterations in host immunity, stress and other factors can lead to *C. albicans* overgrowth, causing a wide range of infections, from superficial mucosal to disseminated candidiasis, including VVC. Among the symptoms of VVC there are pruritus, vaginal soreness, dyspareunia, external dysuria, and abnormal vaginal discharge. During a lifetime, greater than 50 % of women aged 25 years and over, have suffered from VVC at some time, fewer than 5 % of these women experience recurrences. The optimal treatment for recurrent VVC has not yet been defined (Ringdahl, 2000; Workowski and Gail, 2015).

Among antifungal drugs, the class of imidazoles is still considered the first-line treatment for *C. albicans* infections. Clotrimazole (CTZ) is a synthetic derivative belonging to this class of molecules that possess a broad-spectrum antifungal activity against pathogenic dermatophytes and yeasts. It is present in the market in different pharmaceutical form (e.g., creams, tablets, ovules) and its mechanism of action rely on the inhibition of ergosterol synthesis and the alteration of the permeability of the cell wall (Kasper et al., 2015). The common treatment for VVC consists in single or multiple daily topical intravaginal applications of an imidazole cream from 3 to 7 days and, in the case of recurrences, the therapy should be continued with daily oral antifungal drugs up to 6 months, as first line maintenance regimen (Ringdahl, 2000; Workowski and Gail, 2015). Despite CTZ is widely employed in the treatment of VVC, due to its poor solubility, repeated topical administrations are required to maintain a local therapeutic concentration.

Conventional vaginal medications (*i.e.*, solutions, emulsions, creams, etc.) are somewhat effective, but they still present several drawbacks such as i) leakage and messiness; ii) difficulty on providing an exact dose for creams and gels; iii) low retention to the vaginal epithelium; iv) poor patient compliance due to numerous repeated applications (Johal et al., 2016).

Compared to conventional vaginal dosage forms, intravaginal rings (IVRs) offer the possibility of a controlled vaginal drug delivery over a determined period with a single application (Tietz and Klein, 2019). IVRs are flexible devices made with biocompatible polymers with the advantages of safety, local application, few adverse effects, controlled drugs release and moreover, good patient compliance due to a one-time application for a long period of treatment (Fu et al., 2018). The concept of sustained, localized drug administration to the human vagina using a polymeric ring was first

published in 1970 (Mishell and Lumkin, 1970) and since then, seven different rings have been marketed for hormone replacement or contraception (Welsh et al., 2019). Whereas for a long time IVRs have been developed only for hormonal therapy, in recent years there have been an increasing interest on this type of device as promising delivery system for microbicides against HIV, vaginal microbial infections and other sexually-transmitted diseases (Ferguson and Rohan, 2011; Tietz and Klein, 2019; Verstraete et al., 2017).

Among the manufacturing processes for IVRs production, an innovative and still not widely explored method is 3D printing (Fu et al., 2018). Fused deposition modeling (FDM) is a 3D printing technique that allows to print objects layer by layer after a computer assisted design (CAD) using a wide range of materials.

In recent years this technique has emerged in the pharmaceutical field with multiple applications due to the possibility of combining drugs with different thermoplastic polymers using hot melt extrusion (HME) technique to produce the filaments needed to feed the printer (Melocchi et al., 2020).

Compared to conventional IVRs manufacturing techniques (e.g., extrusion or injection molding), with 3D printing, different shapes and dimensions can be easily produced to meet patients' needs and to increase acceptability and adherence (Fu et al., 2018; Morrow Guthrie et al., 2015).

Among materials that can be utilized with FDM, for the manufacture of IVRs, thermoplastic polyurethane (TPU) possesses the necessary characteristics of elasticity and flexibility, as well as a high potential to provide sustained drug release. Moreover, it has already been utilized to produce drug-loaded vaginal devices and marketed IVRs (Domínguez-Robles et al., 2020; McBride et al., 2019; Verstraete et al., 2017).

The aim of this work was to develop an innovative 3D printed IVR loaded with clotrimazole to treat vulvovaginal candidiasis providing higher patience compliance compared to the actual daily treatments. For this purpose, rings were printed using TPU previously loaded with two different concentrations of the antifungal drug CTZ using the FDM technique. After an initial characterization, the drug release from the prepared IVRs was evaluated and then the *in vitro* antifungal activity was investigated against *C. albicans* to confirm the efficacy of the device over a period of 7 days.

2. MATERIALS AND METHODS

2.1 Materials

Thermoplastic polyurethane (TPU) Tecoflex^{TM} EG-100A was kindly gifted from Lubrizol (USA), clotrimazole (CTZ) was purchased from Fluorochem (UK). Sodium chloride, glacial acetic acid, sodium acetate, sodium lauryl sulfate (SLS), formic acid and pure ethanol were provided by Merck (Germany). DL-Lactic acid sodium salt 60% aqueous solution and lactic acid FU-BP were purchased from A.C.E.F. (Italy). All the other solvent used were HPLC grade.

2.2 Preparation of thermoplastic poly(urethane) (TPU) filaments containing clotrimazole (CTZ)

For the fabrication of 3D printed IVRs, FDM feeding filaments were prepared using HME by combining TPU and CTZ. An oil method was used to ensure an homogeneous distribution of CTZ on the pellets' surface, as previously reported (Domínguez-Robles et al., 2020; Mills et al., 2015, 2018). Briefly, 30 g of TPU pellets were placed in a 50 mL conical centrifuge tube and 30 µL of castor oil were added. Next, the tube was vortexed for a few minutes to let the oil homogeneously cover all the pellets and then, the oiled pellets were transferred in a new 50 mL centrifuge tube to avoid wastage of drug that could remain stuck to the excess of oil still attached on the tube wall. At this point, a precise amount of CTZ, depending on the final concentration (2% and 10% w/w), was added and the tube was vortexed again to coat the pellets. The coated pellets were fed in the filament extruder (Noztek Pro HT, 3 mm nozzle, Noztek, UK) and extruded at 190 °C. To ensure homogeneity of the drug, the obtained filament was pelletized and extruded a second time using the same conditions to obtain the final filament with the diameter of 2.85 mm to be effectively printed. Blank IVRs were prepared using pure TPU filament produced by introducing TPU pellets (previously covered with castor oil) directly into the extruder at 190 °C. Composition of TPU filaments containing CTZ are reported in table 1.

Table 1. Composition of TPU filaments containing CTZ

Formulation	TPU (g)	Castor oil (µL)	CTZ (g)
TPU	30	30	0
2% CTZ	30	30	0.9
10% CTZ	30	30	3

2.3 Fabrication of 3D printed IVRs containing CTZ by FDM 3D printing

Drug-loaded and blank IVRs were printed with the previously prepared filaments using an Ultimaker 3 FDM 3D printer (Ultimaker, The Netherlands). The ring model with 54 mm of outer diameter (OD) and 4 mm of cross-sectional diameter (CSD) was designed with a CAD-based software and then converted to a print pattern using Ultimaker Cura 4.6 software (Ultimaker, The Netherlands). The layer height was set at 0.1 mm with 100% of infill density and a printing speed of 25 mm/s. The printing temperature was set at 220 °C with the build plate kept at room temperature.

2.4 Characterization of 3D printed IVRs

After 3D printing of the IVRs, they were weighed and the OD and CSD were measured using a digital caliper (Mitutoyo, Japan). Care was taken to ensure that the IVRs were not compressed or distorted during measurements. The elongation resistance was measured with a customized 3D printed system. To evaluate the homogeneous distribution and the effective amount of CTZ present in each ring, different samples were cut from the printed rings and dissolved in chloroform. Then, the amount of CTZ was measured with an UV spectrophotometer (T60, PG instruments, UK) after the preparation of a calibration curve with a concentration ranging from 0.01 to 0.1 mg/mL. The correlation coefficient (R^2) obtained was 0.991. TPU and CTZ before and after HME and 3D printing were also analyzed by attenuated total reflectance Fourier transformed infrared spectroscopy (ATR-FTIR, Spectrum Two FT-IR spectrometer with ATR accessory, Perkin Elmer, MA, USA). Measurements were performed at 450-4000 cm^{-1} with a resolution of 4 cm^{-1} and a total of 64 scans. Finally, thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) were performed. TGA was performed to measure the weight loss of CTZ, 3D printed TPU, and 3D printed TPU with 10% of CTZ since the elastomer was subjected to high temperatures during the printing process. TGA was performed using a TGA Perkin Elmer 4000 instrument (Perkin Elmer, MA, USA). Scans were run at room temperature to 550 °C, at a speed rate of 10 °C/min under a nitrogen flow rate of 30 mL/min. Differential Scanning Calorimetric (DSC) data were collected using a DSC Perkin Elmer 6000 instrument (Perkin Elmer, MA, USA), calibrated with high purity standards. Approximately 5 mg samples were placed in aluminum pans and were heated up with a fixed heating rate of 5 °C/min from 25 to 200 °C, cooled down at a fixed cooling rate of 5°C/min to -40 °C and heated up again to 200 °C.

2.5 Microbial strain and culture conditions

The reference strain *C. albicans* ATCC 10231 was used in this study. The strain was routinely grown on Sabouraud dextrose agar (SDA) plates (VWR, Milan, Italy), incubated at 37 °C for 24 h. Stock cultures were maintained at -80 °C in nutrient broth (VWR) with 15% of glycerol.

2.6 Minimum Inhibitory Concentration (MIC) determination

MIC were determined by the standard micro-dilution method (Wayne, 2017). Firstly, a CTZ stock solution was prepared in DMSO of biological grade (2 mg/mL) and stored at 4 °C in the dark. Then, 100 µL of *C. albicans* ATCC 10231 suspension (10^6 CFU/mL), was diluted 1:50 in standard RPMI 1640 medium (Sigma-Aldrich, Milan, Italy) and inoculated into a 96-well plate together with the appropriate volumes of CTZ solution (0.0625-16 µg/mL). Two rows were left for control growth (inoculated medium without antifungal agent) and negative control (medium only), respectively. Preliminary assays with DMSO were carried out to exclude its possible antifungal activity; in any case, the volume of DMSO never exceeded 5 % (v/v). Plates were incubated at 37 °C and examined after 24 h. MIC is defined as the lowest drug concentration that inhibits the visible growth in comparison with the control. The turbidity of the 96-well plate was also measured using a spectrophotometer (530 nm) (Multiskan EX, Thermo Scientific).

2.7 Preliminary anticandidal assay in agar plates

At first, all the formulated IVRs (0, 2 and 10% CTZ) were sterilized by UV irradiation under flow safety cabinet for 1 h (30 minutes for each side) and maintained in sterile petri dishes. To perform the anticandidal assay, a modified agar diffusion method was used. Briefly, several colonies of *C. albicans* ATCC 10231 were inoculated into 15 mL of tryptic soy broth (TSB, VWR) and incubated at 37 °C for 24 h. At the end of the incubation period, the microbial suspension was adjusted to *ca* 10^6 CFU/mL (OD 600 nm 0.13–0.15), and 500 µL of this culture was added to 25 mL of liquid sterile SDA maintained at 50 °C; 15 mL were poured into a petri dish and allowed to solidify for several minutes, afterward the formulated IVR was placed on the solidified layer and the remaining 10 mL of inoculated SDA were poured to embed the medical device. This procedure was repeated for each formulated IVR in duplicate. The plates were then incubated at 37 °C for 24 h, and a well-defined zone of growth inhibition visible around each IVR was considered as index of antimicrobial activity.

2.8 *In vitro* clotrimazole release study

The *in vitro* release of CTZ from the 3D printed IVRs was evaluated in two different release media. The first was a 50% ethanolic solution and the second was a vaginal fluid simulant (VFS) (Rastogi et al., 2016) added with 1% w/v of sodium lauryl sulfate (SLS). The addition of SLS was necessary to maintain sink condition due to the very poor solubility of CTZ in aqueous solvents (Saadatfar et al., 2018).

For this study, the 3D printed IVRs were placed in sealed glass bottles with 100 mL of release medium to ensure that sink conditions were maintained. The bottles were then stored in an orbital shaking incubator at 37 °C, 100 rpm for seven consecutive days. During the first day, 1 mL samples were withdrawn every hour for the first 6 hours replacing the volume with fresh medium. Then, every 24 hours, a sample was withdrawn for the analysis and the total volume of release medium was replaced with fresh one. The amount of CTZ released from the IVRs was measured with a high-performance liquid chromatography (HPLC) coupled with a diode array detector (DAD). The drug release from IVRs was evaluated in triplicate.

2.9 HPLC-DAD method for the analysis of CTZ

The amount of CTZ released during the *in vitro* studies was evaluated by HPLC (Agilent 1260 Infinity II, Agilent, USA) using a mixture of 0.5% formic acid in water and acetonitrile (ratio 55:45 for EtOH 50% release medium and 45:55 for VFS + 1% SLS release medium) as mobile phase, with a flow rate of 1 mL/min in an Agilent Zorbax Eclipse Plus C18, 150 x 4.6 mm, 5 µm column (Agilent, USA). The injection volume was 20 µL and the detection signal was recorded at 230 nm (UV lamp) keeping the analysis system at room temperature. Two different calibration curves of CTZ were performed with a concentration ranging from 0.005 to 0.2 mg/mL in EtOH 50% and in VFS + 1% SLS. The correlation coefficient (R^2) obtained was 0.9996 for both curves.

2.10 Time-kill assay

The time-kill studies curves allow to assess the exposure time required to kill a standardized *Candida* inoculum. In this study, vaginal simulative medium (VSM) (Kasper et al., 2015) was used to simulate the typical environment of *Candida* infection in women. The composition of VSM was the following: bovine serum albumin (18 mg/L), NaCl (3.5 g/L), KOH (1.4 g/L), $Ca(OH)_2$ (0.22 g/L), lactic acid 90% (2.2 g/L), glycerol 50% (0.32 g/L), urea (0.4 g/L), glacial acid acetic (1 g/L), glucose (0.5% w/v) adjusted to pH 4.2. The medium was sterilized by 0.22 µm filter and maintained at 4 °C before use.

Two days prior the experiments, a series of sterile tubes containing 20 mL of VSM were prepared according to the following scheme: one tube with one 10% CTZ-IVR was incubated for 48 h at 37 °C (48h conditioned VSM 10% CTZ-IVR) with gentle shaking (100 rpm) and one tube with one 10%

CTZ-IVR was incubated for 24 h at 37 °C (24h conditioned VSM 10% CTZ-IVR) with gentle shaking (100 rpm). The test organism *C. albicans* ATCC 10231 was incubated in 15 mL of TSB at 37 °C for 24 h. At the end of the incubation period, the microbial suspension was centrifuged at 3500 rpm for 10 min, the pellet was resuspended in the same volume of VSM and the turbidity was adjusted to *ca* 10^6 CFU/mL (OD 600 nm 0.15). 1 mL of this inoculum was added to the different sterile tubes containing 20 mL of VSM and the formulated IVRs, while in the unconditioned VSM sample, the 10% CTZ-IVR was added simultaneously with *C. albicans*. Lastly, one sterile tube containing 20 mL of VSM was inoculated as control growth together with one 0% CTZ-IVR. All the tubes were incubated at 37 °C with gentle shaking (100 rpm) and, at established time points (up to seven days: baseline, 24, 48, 72, 96, 120, 144 and 168 h), 100 µL aliquots were aseptically removed from each tube, serially diluted in sterile physiological saline solution and spread in triplicate (10 µL) onto SDA plates. After 24 h of incubation at 37 °C, the plates were observed and CFU were enumerated. All the experiments were performed three times using independent cultures.

3. RESULTS AND DISCUSSON

3.1 Fabrication and characterization of 3D printed IVRs

The materials utilized for the fabrication of IVRs require different mechanical characteristics such as elasticity and flexibility. The most common utilized ones are silicone elastomer, ethylene-vinyl acetate (EVA) and polyurethane (Boyd et al., 2019; Johal et al., 2016). In recent years, thermoplastic polyurethane (TPU) has shown great potential for the manufacturing of sustained release matrices and it has already been successfully employed in the development of vaginal devices and in 3D printing (Domínguez-Robles et al., 2020; Verstraete et al., 2018). It is biocompatible and possesses the required physicochemical and drug release properties needed for the development of IVRs (Malcolm et al., 2016; Verstraete et al., 2017; Welsh et al., 2019).

In this study, we intended to prepare IVRs for controlled release of CTZ using FDM 3D printing technology. In order to feed the printer, TPU pellets mixed with two different concentrations of CTZ (*i.e.*, 2% and 10% w/w) were extruded via HME to produce smooth and flexible filaments with a diameter of 2.85 mm. The prepared filaments were clear, transparent with a light yellowish color increasing in intensity with the CTZ concentration.

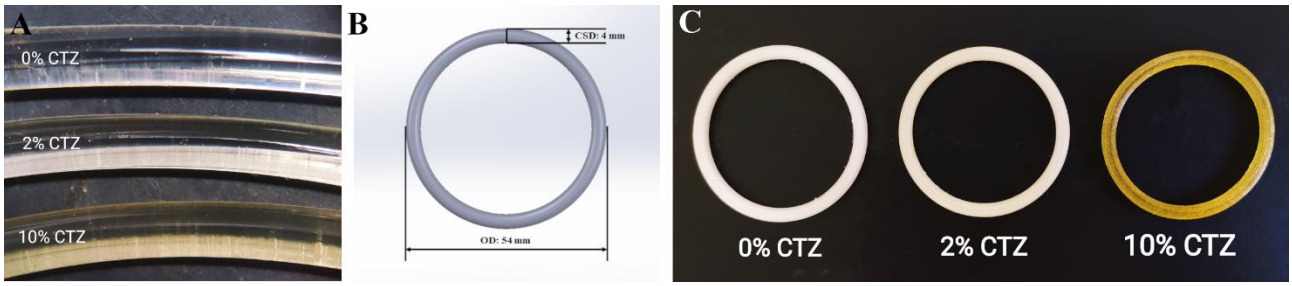

Figure 1. Fabrication of 3D printed IVRs: A) TPU filaments produced via HME loaded with different concentrations of CTZ; B) CAD 3D image of the IVR; C) 3D printed IVRs loaded with CTZ.

Using a stereo microscope (Nikon SMZ-1, Nikon, Japan), no visible aggregates of the drug were seen within the extruded materials (Figure 1A). An effective mixing was also due to the melting of CTZ during the extrusion process that ran at 190°C meanwhile the melting temperature of the drug is between 136.8 and 153.1 °C (Garcia Ferreira et al., 2019). Moreover, to reach a more homogeneous mixing, the filaments were cut again in pellets and extruded a second time. As previously reported (Domínguez-Robles et al., 2020), we confirmed again that it is possible to properly mix TPU and CTZ using a single screw extruder following the pellet coating oil method, avoiding the utilization of more advanced equipment such as a twin-screw extruder.

The prepared TPU filaments were used to feed the printer and prepare the IVRs by FDM technique. The ring was designed using a CAD software complying with the measures of commercial thermoplastic rings *i.e.*, an OD of 54 mm and a CSD of 4 mm (Figure 1B). Figure 1C shows the 3D printed IVRs produced with 0%, 2% and 10% of CTZ TPU filaments. The drug-loaded prototypes presented a yellowish color due to the presence of CTZ as already noticed during the production of the filaments. Immediately after printing, the rings were measured for their OD and CSD taking care not to compress or distort them during measurement and then weighted as reported in table 2. All the manufactured rings were considered dimensionally accurate as they were within the specific acceptance criteria.

Table 2. Dimensional and mass analysis of produced IVRs

Sample	Mass (g)	CSD (mm)	OD (mm)
TPU-IVR	2.162±0.034	3.98±0.07	53.98±0.21
2% CTZ-IVR	2.175±0.021	4.05±0.05	54.03±0.17
10% CTZ-IVR	2.351±0.032	4.03±0.02	54.01±0.15

To evaluate the homogeneous distribution and the amount of CTZ present in the rings, different pieces were cut and weighted from the printed IVRs and then dissolved in chloroform to measure the amount

of the active molecule with an UV spectrophotometer. The average amount of CTZ was 66.2 ± 1.1 mg/g of ring proving an effective CTZ loading of 6.6%. The average weight of printed IVRs was 2.286 ± 0.101 g with an average amount of 151 mg of CTZ for one ring.

To establish whether any interaction had occurred between TPU and CTZ, the materials were analyzed by FTIR before and after HME, and then after 3D printing (Figure 2). FTIR spectra of raw materials presented the typical peaks (Gupta et al., 2013; Haryńska et al., 2018) meanwhile any change in the peaks were noticed after the incorporation of the drug into the thermoplastic polymer suggesting that there were no new chemical bonds formed for the TPU during extrusion or printing processes.

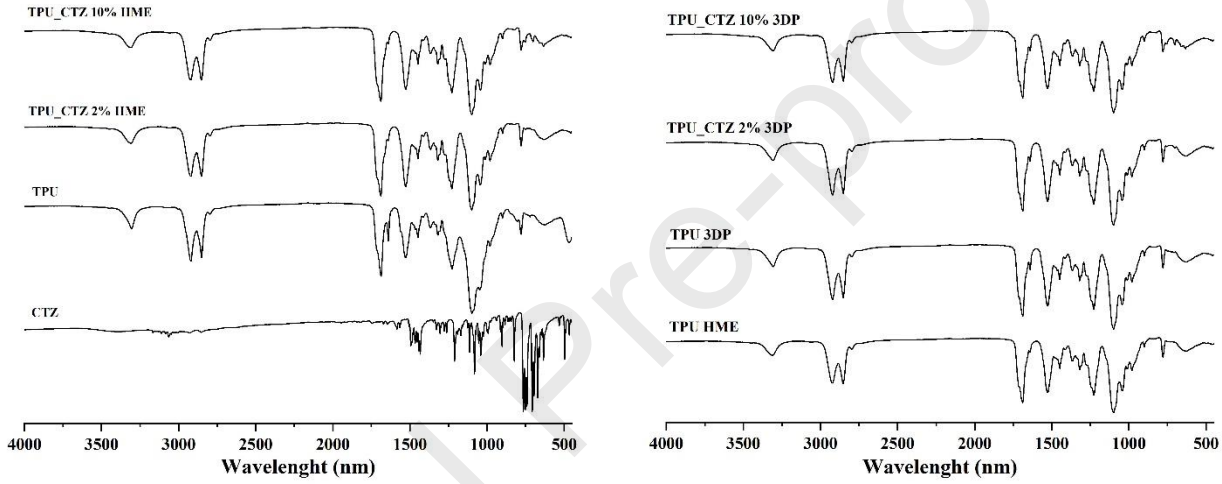

Figure 2. FTIR spectra of CTZ, TPU and their blends after hot melt extrusion (HME) and 3D printing (3DP).

As thermal stability is a necessary condition for candidate drugs to be safely utilized in HME, TGA measurements were performed confirming that CTZ stays stable at processing temperatures with a weight loss lower than 3% at 220 °C (Figure 3A, B) (Garcia Ferreira et al., 2019). When CTZ was combined with TPU, the resulting material presented different thermal degradation behaviour. For pure TPU, the T_{onset} (5% weight loss) of the thermal degradation falls at about 316°C and it drops to a lower temperature (297°C) in the presence of CTZ. Moreover, the DTG curves (Figure 3C, D) revealed that the presence of CTZ reduces the temperature related to the maximum degradation rate of the pure TPU suggesting the CTZ causes a partial degradation of the TPU.

This temperature differences can be attributed to interactions between the TPU and the CTZ.

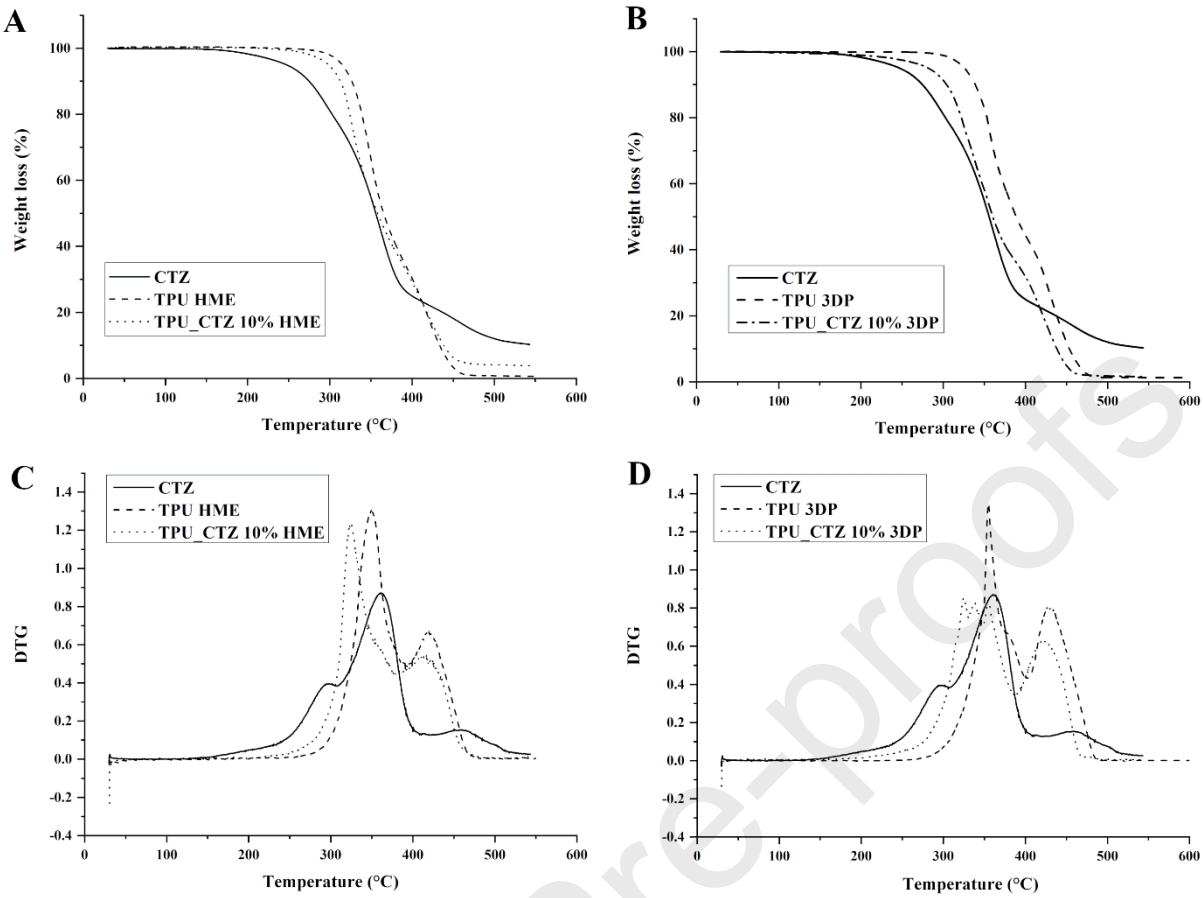

Figure 3. (A) TGA after HME, (B) TGA after 3DP, (C) DTG after HME, and (D) DTG after 3DP of CTZ, TPU, and TPU containing 10% of CTZ.

DSC measurements reported in figure 4 showed no changes in the glass transition temperatures (T_g) of pure TPU and TPU containing 10% of CTZ indicating that the active molecule did not act as plasticizer.

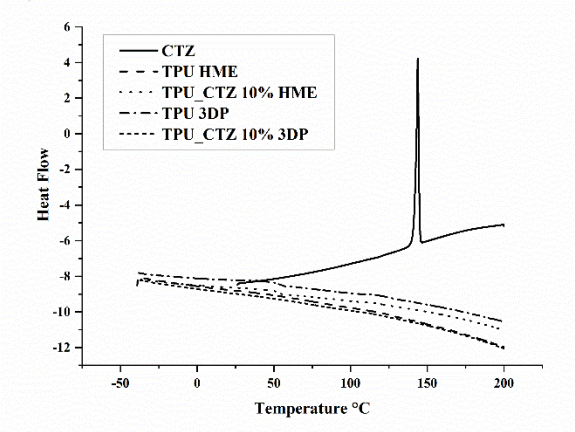

Figure 4. DSC curves of CTZ, TPU, and TPU containing 10% of CTZ after HME and 3D printing.

Finally, to measure the elongation capacity of the 3DP IVRs, a customized 3D printed tensile testing jig with specifications reported elsewhere (McCoy et al., 2019) was used. Tensile elongation is commonly used as a test method to assess the ultimate tensile strength of an IVR. It may reflect ring removal from the vagina, where a user hooks a finger around the inner diameter and pulls firmly to remove the device (McCoy et al., 2019). All the fabricated IVRs reached an acceptable elongation higher than 300% without breaking (Verstraete et al., 2017).

3.2 Antifungal preliminary studies

The results relative to the anticandidal activity of IVRs (CTZ 2 and 10%) and relative control (CTZ 0%) are presented in figure 5.

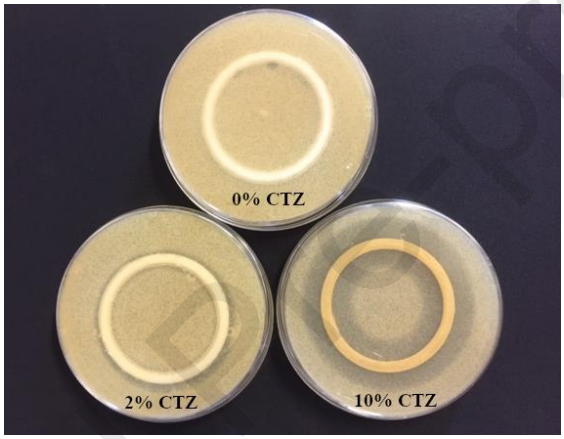

Figure 5. Preliminary antifungal activity of prepared IVRs, assessed by a modified agar-diffusion method.

The presence of the formulated IVRs resulted in a different inhibition of *C. albicans* ATCC 10231 growth. As shown in figure 5, in the plate with the highly CTZ loaded IVR (10% CTZ), the area of growth inhibition reached 2 cm (considering the IVR itself) compared to the negligible one observed for the IVR with 2% CTZ. The control TPU IVR without antifungal agent showed no growth inhibition. These preliminary studies suggested a possible effective antifungal activity of the 10% CTZ-IVR, that was then chosen for the following studies.

3.3 *In vitro* drug release studies

Whereas most of the currently marketed vaginal dosage forms are designed as immediate release formulations (*i.e.*, creams, gels, tablets, etc.), IVRs offer the possibility of a controlled vaginal drug delivery for a prolonged period of time with only one application. The IVR developed in this work is considered a matrix-type device in which the drug is homogeneously dispersed/dissolved in the matrix polymer and, drug release rates are proportional to both the drug loading and the surface area

of the device (Malcolm et al., 2010). A compendial dissolution method does not exist since monographs for IVRs are not present in the major international pharmacopoeia.

In this work, to evaluate drug release from IVRs, we utilized the shake-bottle method that was performed in an incubator shaker in which sealed bottles containing the IVRs and a specific volume of a pre-heated dissolution medium were placed. During this type of studies, it is essential to choose the appropriate dissolution medium for ensuring sink condition throughout the experiment. The use of vaginal fluid simulant media with physiological vaginal pH and osmolarity, represents a first setup towards physiologically relevant dissolution media for IVRs taking conditions as biorelevant as possible (Boyd et al., 2019; Tietz and Klein, 2019). For this reason, VFS was chosen with the addition of 1% of SLS (Boyd et al., 2014) to maintain sink conditions due to the poorly soluble drug used. Together with this medium, we also tested the drug release in a mixture of 50% ethanol in water (McConville et al., 2015) as solvent/water mixtures are recently become common for highly water-insoluble drugs (Boyd et al., 2019). Despite organic solvent mixtures are not as biologically relevant as media that simulate vaginal fluid, their utilization could be applied in quality control (QC) that normally requires discriminating and robust methods in which a biopredictivity is not required.

In figure 6, the cumulative and daily CTZ releases from the 3D printed 10% CTZ-IVRs are reported.

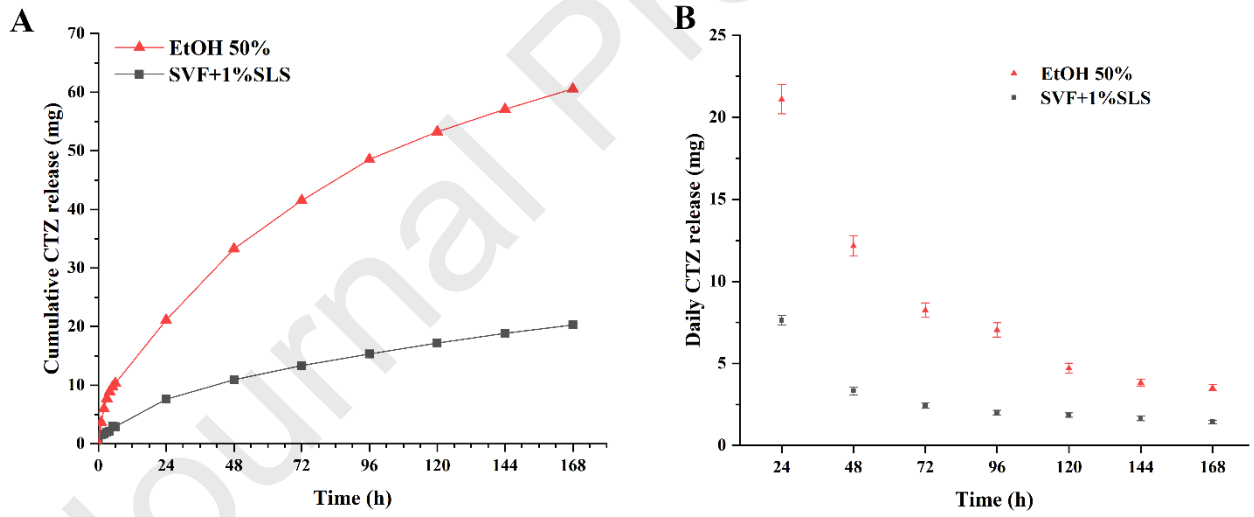

Figure 6. Drug release from 3D printed 10% CTZ-IVRs. (A) Cumulative CTZ release; (B) Daily CTZ release (mean ± SD, n=3)

The daily CTZ release ranged from 1.43 to 7.63 mg in the VFS and from 3.51 to 21.11 mg in the 50% EtOH medium showing a sustained release of the drug during seven days in both the tested media. The cumulative release resulted in 20.29 mg in VFS and 60.60 mg in 50% EtOH after one week (13.4% and 40.1% of the total amount present in one ring respectively). The amounts released were

well above the MIC value measured for *C. albicans* (0.0625 µg/mL) therefore, despite these results, the antifungal effectiveness was also tested *in vitro*.

In release studies, mathematical models play a crucial role in evaluating the drug release mechanism (Siepmann and Peppas, 2011). For matrix-type ring, like the one developed in this work, drug release under sink conditions normally adhere to the so-called "root-time kinetics". This is confirmed by a linear plot when the cumulative release data is plotted against the square root of time, a model first introduced by Higuchi in 1961 (Higuchi, 1961).

Here, the CTZ release data were fitted using the Higuchi equation giving a high linearity (R^2) of 0.998 in both the release media confirming the diffusion kinetic already reported in many other matrix-type IVRs (Boyd et al., 2019; Malcolm et al., 2003; Murphy et al., 2018; Ugaonkar et al., 2015).

3.4 *In vitro* effectiveness of CTZ-IVRs against *C. albicans*

The examined strain exhibited sensitivity to CTZ, showing MIC value of 0.0625 µg/mL. It was in accordance to those reported in the literature for *Candida* species isolated from vaginal infection (Nagashima et al., 2016).

Based on the antifungal preliminary data in agar, only the IVRs with 10% CTZ were used for the time-kill experiments in VSM. The antifungal effect of the formulated IVRs was confirmed on the tested *C. albicans* strain with an increased rate of microbial growth reduction during the entire incubation time (from 24 to 168 h) (Figure 7).

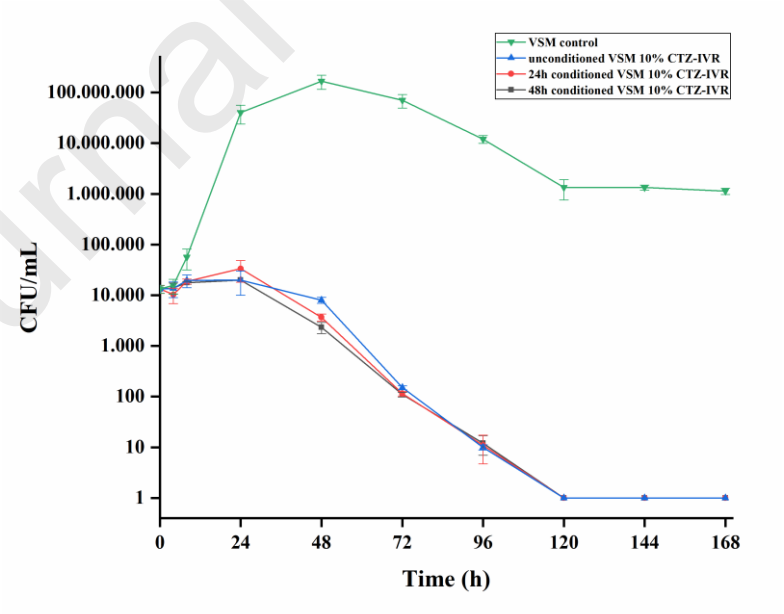

Figure 7. *C. albicans in vitro* time-kill experiment with 10% CTZ-IVRs pre-incubated for 24 and 48 h, incubated together with the fungi at time 0 and control incubated with 0% CTZ-IVRs.

In the performed experiments, the IVRs were left in VSM for 24 and 48 h to allow the release of CTZ in the medium prior to the addition of *C. albicans* ATCC 10231 suspension to simulate also a possible preventive antifungal application. As shown in figure 7, after 24 h of incubation, the viability of *C. albicans* decreased to $2x10^4$ CFU/mL in the 48h conditioned VSM CTZ-IVR and to *ca* $3x10^4$ CFU/mL in the 24h conditioned VSM CTZ-IVR, similar to the decrease ($2x10^4$ CFU/mL) observed in the unconditioned VSM CTZ-IVR. In all the IVRs-containing samples, after 24 hours the CFU/mL values were much lower compared to $4x10^7$ CFU/mL of the VSM growth control sample (with 0% CTZ-IVR) suggesting that the rings could be effective both in preventive and maintenance applications. The antifungal activity could be attributed only to the release of the drug since, in the VSM control containing the 0% CTZ TPU IVR, *Candida* growth was uncontrolled. In the following time points (from 48 to 96 h) a more drastic decrease of *C. albicans* viability was observed in all the CTZ-IVR samples (values ranging from $1.5x10^2$ to 10 CFU/mL), reaching the complete growth inhibition (no detectable CFU/mL) after 120 h of incubation.

From the analysis of the data, we can observe that the viability reduction caused by the formulated IVRs was not related to the conditioning of the VSM (for 24 and 48 h), probably because the daily CTZ released in the medium (Figure 6) resulted higher than CTZ MIC value. It can be noted that VSM presents features mimicking the physiological conditions during *C. albicans* growth in the vagina (low concentration of organic compounds, acid pH) (Kasper et al., 2015), and this aspect is fundamental to have a better biopredictivity of the efficacy of the 3D printed IVRs.

4. CONCLUSIONS

For the first time, we have been able to produce 3D printed IVRs loaded with CTZ using FDM technique. The results obtained from *in vitro* studies against *C. albicans* are encouraging for possible applications against fungal infections. The drug release and time-killing studies were performed using media simulating the vaginal fluid to be more biorelevant.

As the common treatment for fungal infection consists in multiple applications of conventional dosage forms, the utilization of a vaginal device such the CTZ-loaded ring could improve the patience compliance by decreasing the number of applications to one only switching from daily to weekly therapy. Moreover, the 3D printing technique allows the production of different shapes and sizes that can be helpful to have a personalized device based on the patient needs. Its utilization could be considered for immediate treatment perhaps together with conventional formulations and/or for maintenance therapy in case of recurrences instead of daily oral antifungal therapies.

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Table 1. Composition of TPU filaments containing CTZ

Formulation	TPU (g)	Castor oil (µL)	CTZ (g)
TPU	30	30	0
2% CTZ	30	30	0.9
10% CTZ	30	30	3

Table 2. Dimensional and mass analysis of produced IVRs

Sample	Mass (g)	CSD (mm)	OD (mm)
TPU-IVR	2.162±0.034	3.98±0.07	53.98±0.21
2% CTZ-IVR	2.175±0.021	4.05±0.05	54.03±0.17
10% CTZ-IVR	2.351±0.032	4.03±0.02	54.01±0.15

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Mattia Tiboni: Conceptualization, Methodology, Formal analysis, 425 Investigation, Data curation, Writing - original draft.
Raffaella Campana: Methodology, Formal analysis, Investigation, Data curation.
Emanuela Frangipani: Methodology, Data curation, Writing - review & editing.
Luca Casettari: Conceptualization, Resources, Funding acquisition, Project administration, Supervision, Writing - review & editing.