Novel drug-loaded film forming patch based on gelatin and snail slime

NOVEL DRUG-LOADED FILM FORMING PATCH BASED ON GELATIN AND SNAIL SLIME

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Abstract

Gelatin-based films enriched with snail slime are proposed as novel biodegradable and naturally bioadhesive patches for cutaneous drug delivery. Films (thickness range 163-248 μm) were stretchable and they adhered firmly onto the wetted skin, especially those with high amount (70% V/V) of snail slime extract. Fluconazole, was selected as model drug and added to films containing the highest amount of snail slime. The presence of Fluconazole (4.53±0.07% w/w) did not modify significantly the mechanical properties, the swelling degree and the bioadhesive performances of the films. Structural investigations demonstrated that the crystalline form III of the drug changed to the amorphous form, forming an amorphous solid dispersion. Moreover, snail slime prevented the drug recrystallization over time. *In vitro* permeation studies showed that film exhibited a cumulative drug concentration (over 60% in 24 h) similar to that of the control solution containing 20% w/V of ethanol. Fluconazole-loaded gelatin films proved to be effective towards clinical isolates of *Candida spp.* indicating that the drug maintained its remarkable antifungal activity once formulated into gelatin and snail slime-based films. In conclusion, snail slime, thanks to its peculiar composition, has proved to be responsible of optimal skin adhesion, film flexibility and of the formation of a supersaturating drug delivery system able to increase skin permeation.

Keywords: gelatin; snail mucus extract; Fluconazole; amorphous solid dispersion; bioadhesive films; skin penetration.

1. INTRODUCTION

In the last decades, thin polymeric films have been proposed as an alternative approach to conventional pharmaceutical forms such as creams, ointments and gels for the local delivery of drugs, both to mucosal tissue (Dixit and Puthili, 2009; Morales and McConville, 2011; Silva et al., 2015) (either as mucoadhesive films or as orodispersible films) and to skin (Silva et al., 2008; Padula et al., 2019; Voss et al., 2020). Polymeric thin films can also be un-medicated (Shanmugapriya et al., 2018) or drug impregnated (Maver et al., 2019) for use as a wound dressing.

Topical formulations for the local delivery of drugs must provide an effective concentration and a controlled release to avoid multiple applications per day; furthermore, they must have non-irritant ingredients and be sensitive to the skin, compatible with the acid mantle (Ali and Yosipovitch, 2013). Films, intended as topical patches, are solid flexible preparations which are able to deliver accurate dosing of drug and generally include all the components (drug, polymeric matrix, plasticizer, adhesive) in one layer of material (Padula et al., 2009). Unlike common topical patches (medicated plasters), films do not need of an appropriate support, usually made of synthetic material, and can be designed either to be easily washed off or to resist water and to be self-adhesive (Lieb et al., 2002) or to adhere to wet skin (Padula et al., 2007). Moreover, their relatively high-water content is important in skin moisturization and elasticity, providing a better feeling when applied to the skin (Silva et al., 2008). The type of polymer used for film preparation and its properties (e.g., molecular weight, degree of substitution, ionizable groups) may influence the characteristics of films such as adhesive properties, mechanical strength, water uptake ability, drug solubility and drug release rate. For all these reasons, films offer a versatile platform as carrier of a variety of drugs for topical delivery.

Among the most used polymers for the production of films (cellulose derivatives, poly vinylpyrrolidone, polyvinyl alcohol, polyethylene oxide, pullulan, pectin and chitosan), gelatin is a biodegradable polypeptide of natural origin, obtained by the controlled partial hydrolysis of collagen (Karki et al., 2016; Marfil et al., 2012). Gelatin displays several attractive features, such as absence of antigenicity, low cost, biocompatibility

and adhesiveness (Gómez-Guillén et al., 2011; Mano et al., 2007). The capability of gelatin to form films is well known (Ramos et al., 2016; Hassan et al., 2018; Dolci et al., 2018; Dolci et al., 2020), and a variety of compositions and blends have been investigated (Boanini et al., 2010; Amadori et al., 2015).

Snail slime has currently revolutionized the world of cosmetics and skincare, although it has been used in medicine from ancient times for pain relief, treatment of injuries due to the combination of natural ingredients with beneficial therapeutic qualities for human skin. Snail slime composition depends on several variables as snail species, snail feeding and extraction method and mainly includes, among others, allantoin, glycolic acid, collagen and several mucin-like glicoprotein complexes (Tsoutsos et al., 2009; Lopez Angulo and do Amara Sobral, 2016; Zhong et al, 2018 and Gubitosa et al., 2020). Recently, the authors have patented (Albertini et al., PCT/EP2020/059427) the use of Snail slime as a component of film-forming solutions with a number of biopolymers and found that snail addition strongly influenced chitosan and cellulose derivatives-based films (Di Filippo et al., 2020; Di Filippo et al., 2021), conferring them unusual but desirable properties. In particular, snail mucus addition to both chitosan-based and cellulose-based films provided them with antimicrobial activity against both Gram-positive and Gram-negative bacteria, remarkably improved their water barrier and bioadhesion properties, film elongation, adhesion strength and UV-screening effect. These interesting results indicate that these biocomposite and biodegradable films might be useful in the biomedical field and very good candidates for food packaging as well.

Herein, gelatin-based films enriched with snail slime are proposed as a novel biodegradable and naturally bioadhesive patches for cutaneous drug delivery. In this work, Fluconazole, a broad-spectrum bis-triazole approved in 1990 by the Food and Drug Administration (US FDA), was selected, thanks to its high antifungal activity, which make it one of the most commonly treatment option for virtually all forms of Candida infections in both immune competent and immune compromised hosts. The administration of Fluconazole directly on the skin provides an effective alternative to oral administration and hypodermic injection and reduces the risk of systemic side effects (Bachhav et al., 2011; Gupta and Vyas, 2012; Paolicelli et al., 2017).

In the case of fungal skin infections, an effective drug concentration must penetrate into the stratum corneum, which is responsible for drug low permeability. Fluconazole is less lipophilic when compared to other antifungal imidazole-derivatives, due to the presence of two triazole rings and presents high affinity for keratin prolonging its retention in the skin (Ayub et al., 2007). Therefore, the main obstacle facing the efficiency of topical Fluconazole delivery is the stratum corneum permeation (El-Housiny et al., 2018). The films prepared by mixing gelatin and snail slime were obtained through solvent casting and then characterized by means of FTIR, X-ray spectroscopy and optical and electronic microscopy. Additionally, their swelling properties, tensile strength and adhesion to skin were evaluated. Finally, the ability of promoting the percutaneous absorption of Fluconazole and the antibacterial properties of the novel patch-like platform were assessed.

2. EXPERIMENTAL PART

2.1 Materials and Methods

Porcine gelatin (type A, 280 Bloom) was purchased from Sigma Aldrich. Snail Mucus extracted from *Helix Aspersa Muller* by MullerOne method (http://www.mullerone.com/it/en/extraction-process) was kindly offered by "I Poderi Farm" (Montemerano, Italy) and stored at 4°C. Analysis of Snail mucus was obtained from the supplier and its characteristics along with its main constituents are reported in Table S1 (Supplementary Information). Fluconazole (F) compliant with Ph. Eur. specifications (slightly water soluble: 4,363 mg/L, with a molecular weight of 306.27 Da, log P of 0.5 and a pKa value of 3.7) (Dash et al, 2001) was purchased from Farmalabor (Assago, MI, Italy). Glycerol was purchased from Fagron (Bologna, Italy).

2.1.1 Preparation of gelatin films

Gelatin films (5% w/V) were prepared by dissolving the weighted amount of gelatin in distilled water at 38°C under gently stirring until complete dissolution of the polymer. Then 8.6 mL of this solution were poured in

polyethylene Petri dishes (Ø= 5.5 cm) and kept under laminar flow hood at room temperature overnight. The obtained films were labeled G. Films containing glycerol (30% w/w with respect to gelatin) were prepared by adding it after the dissolution of the polymer, and they are indicated as G_g. All the films were stored at room temperature between plastic-coated aluminum foils inside PVC bags.

2.1.2 Preparation of gelatin-snail slime films

Gelatin films (5% w/V) with different percentages in volume of Snail Slime (S) were produced by adding proper volumes of S to gelatin aqueous solution (obtained through gelatin dissolution in water at 38°C) under stirring. Before addition, the pH of the snail slime was raised to 4.5 using few drops of concentrated NaOH. Different volume of S with respect to the total volume of the films forming solutions (40, 50, 60 and 70% of S) were employed in the preparation of the composite films, which were labeled G_S40, G_S50, G_S60, G_S70, respectively. Then, 6 mL of each solution were poured in Petri dishes (Ø = 5.5 cm) and put under laminar flow hood overnight.

In order to provide a better flexibility and stability over time, glycerol (30% w/w with respect to gelatin) was introduced in the compositions of some selected films after the dissolution of the polymer and the obtained films were labeled by adding "_g" to the above stated tags.

2.1.3 Preparation of gelatin-snail mucus films with Fluconazole

The film composition corresponding to G_S70_g, was chosen for the encapsulation of the antimicrobial drug Fluconazole (F). An amount of 50 mg of Fluconazole (5% weight with respect to the total dry mass of gelatin and S; 10% weight with respect to gelatin) was added to 7 mL of snail slime (adjusted to pH 4.5), stirred for about 2 hours and then mixed with 3 mL of gelatin solution (5% m/V) and glycerol (30% w/w on gelatin weight). Then, 8.6 mL of the mixed solution were poured in polyethylene Petri dishes (Ø= 5.5 cm) and put under laminar hood at room temperature overnight. The obtained films were labeled G_S70_g_F. The obtained films were stored at room temperature between two sheets of plastic-coated aluminum closed inside PVC bags. The compositions and labels of all the obtained films are reported in Table 1.

Table 1. Compositions and labels of the obtained samples.

Labels	Snail Mucus % (v/v)	Water % (v/v)	Glycerol % (w/w) *	Fluconazole % (w/w) **
G	0	100		
G_g	0	100	30	
G_S40	40	60		
G_S50	50	50		
G_S60	60	40		
G_S70	70	30		
G_S70_g	70	30	30	
G_S70_g_F	70	30	30	4.76

* weight of glycerol with respect to the weight of gelatin

** weight of Fluconazole with respect to the dry weight of gelatin, S and Glycerol.

2.2 Films Characterization

2.2.1 Thickness

The thickness of the films was measured with a hand-held digital micrometer (Mitutoyo, Japan) to an accuracy of 0.001 mm. The reported values are a mean of at least five measurements for each sample.

2.2.2 Tensile tests

Tensile tests were performed on films immediately after drying using a 4465 Instron dynamometer equipped with a 100 N load cell and the Series IX software package and stress-strain curves were collected. The Young's modulus (E), the stress at break (σ_b) and the strain at break (ε_b) were evaluated. The test was performed at a crosshead speed of 5 mm/min on strip-shaped samples (40 mm long and 4 mm width). At least 6 specimens were tested for each composition.

2.2.3 Structural Characterization

FTIR spectra were recorded in ATR mode using a Thermo Scientific Nicolet iS10 FTIR spectrometer. Spectra were acquired at room temperature with a resolution of 2 cm^{-1} from 4000 to 800 cm^{-1}.

X-ray diffraction patterns were recorded in the 2θ range from 4° to 40° with a step size of 0,067° and time/step of 40s by means of a Philips X'Celerator diffractometer equipped with a graphite monochromator in the diffracted beam. CuKα radiation at 40 mA and 40 kV was used. Thermogravimetric analysis was carried out using a Perkin-Elmer TGA-7. Heating was performed in a platinum crucible in air flow (20 mL/min) at a rate of 10°C/min up to 800°C. Samples weights were in the range of 5–10 mg.

2.2.4 Swelling measurement

Square-shaped (1cm × 1cm) air-dried gelatin-based films were weighted and immersed in 5 mL of Phosphate Buffer solution (PB) pH 4.5. After set periods of time samples were removed from the solution, wiped with filter paper to remove the excess of liquid and then reweighted. The amount of adsorbed water was calculated as follows:

$$\text{Swelling (\%)} = \frac{Ww - Wd}{Wd} \cdot 100 \quad (1)$$

where Ww and Wd are the weights of the wet and the air-dried sample, respectively.

2.2.5 Bioadhesive properties

The bioadhesive properties of the gelatin films were evaluated by means of an AntonPaar modular compact rheometer MCR102and the RheoCompass Software, as reported in literature (Di Filippo et al., 2020). Pig's ear skin, bought in a local butcher's shop, was carefully separated from the underlying tissue and repeatedly washed with water. Then, disks of 2.5 cm of diameter were cut and glued on the disposable support of the instrument. The film was allowed to adhere on the skin by wetting it with 40 µL of water and applying a gentle finger pressure. Subsequently, the upper plunger, coated with double-sided tape (3M), was lowered until a force of 5 Newton (N) was applied to the film for 30 sec. Then, the plunger was raised at a speed of 1 mm/s, and the force required to detach the film from the skin was measured and expressed in Newton (N). Tests were carried out in triplicate for each composition.

2.2.6 Solubility studies

Solubility measurements at equilibrium of F in different media were performed both at 25°C

(preparation of the sample solutions) and at 32°C (permeation studies). An excess of the drug was added to 5 mL of the following media: phosphate buffer solution (pH 5.5) and phosphate buffer solution (pH 5.5) containing 20% w/V of ethanol. The samples were magnetically stirred for 48 h, equilibrated for 2 h, and the suspensions were then centrifuged at 10.000 rpm for 10 min. The supernatant was filtered through a 0.20 μm membrane nylon filter. After suitable dilutions, the samples were analyzed by HPLC. Each sample was analyzed at least in triplicate.

2.2.7 Analysis of the drug content into the films

Films of about 45 mg were solubilized in 10 mL of a mixture of MeOH: H_2O (40:60 V/V) and left under stirring for 24 h. The samples were filtered and 1 mL of each solution was centrifuged at 8000 rpm for 10 minutes, after which the supernatant was analyzed by HPLC. The samples were analyzed in triplicate and the results of the drug content were expressed as % w/w (weight of F with respect to the weight of the films) and in mg/cm^2.

2.2.8 Fluconazole assay

For the quantitative analysis of F, an HPLC-UV/Vis equipped with LC-10ADvp pumps (Shimazdu), SP-10Avp UV-Vis detector (Shimazdu, 210 nm) and a SIL-20A autosampler (Shimazdu) with a C18 apolar column (Luna Phenomenex, 150 mm x 4.6 mm x 5μm) as stationary phase was used. The mobile phase was composed of acetonitrile 25% and MQ water 75% fluxed at 1 mL/min and with an injection volume of 20 mL. The quantification of F was assessed by means of a calibration curve (r^2 = 0.9999, n=3) made with standard solutions of known concentration in the range 0.5-50 μg/mL. The limit of detection (LOD) and the limit of quantification (LOQ) resulted in 0.18 μg/mL and 0.48 μg/mL, respectively. For the permeation experiments, the same HPLC method was used and quantification was assessed using the same calibration curve.

2.2.9 In vitro skin permeation and retention studies

In vitro permeation experiments were performed to determine the amount of drug able to diffuse from the formulation across the skin. Pig ear skin was selected as membrane due to its similar histological and physiological characteristics and close permeability properties to human skin (Gennari et al., 2020). A Franz-type static glass diffusion cell (15 mm jacketed cell with a flat ground joint and clear glass with a 12 mL receptor volume; diffusion surface area = 1.77 cm^2), equipped with a V6A Stirrer (PermeGearInc., Hellertown, PA, USA) was employed. In the donor compartment, circular films (Ø = 1.1 cm diameter containing 4,76% w/w of F) were placed on pig ear skin (thickness 1,04 ± 0,07 mm) used as membrane. The skin used was obtained from the ears of different pigs, which were kindly provided by a local slaughterhouse (Bologna, Italy). The skin was used after removing the underlying fat and subcutaneous tissues with a surgical blade and stored at −20°C until use (Simon et al., 2016). Each membrane was conditioned for 15 min in PB solution and then carefully placed in the interface between the donor and receptor compartments. The receiver medium contained phosphate buffer solution at pH 5.5 containing 20% of ethanol w/V to prevent F precipitation and it was maintained under stirring at a constant temperature of 32 ± 0.5 C through thermostatic bath circulation (Alberti et al., 2001). The chambers were held together tightly with a cell clamp and sealed with parafilm to limit evaporation. Aliquots of 250 µL were collected at 15, 30, 60, 120, 180, 300 and 1440 min. Sink conditions were maintained with the replacement of the same volume of receptor medium. For comparison, a control solution (0.5 mL) containing the same amount of drug loaded into the film, prepared dissolving the drug in ethanol and then mixed with phosphate buffer solution at pH 5.5, was added to the donor chamber. All collected samples were analyzed by HPLC-UV/Vis and the results of permeation studies are shown as cumulative drug amount permeated per unit of area plotted as a function of time. Each sample was analyzed in quintuplicate and the data are expressed as mean ± SD.

At the end of the permeation study, excess formulation was removed and the skin surface was washed 3 times with PB. The skin samples were cut into small pieces and stored in vials containing 5 mL of methanol

for 1 week. The resulting solution was filtered (0.2 μm), properly diluted (1:10) and the drug amount extracted from the skin was determined by HPLC. The mean of three replicates for each formulation was calculated and the corresponding values were normalized on the average thickness of the skin used in each permeation test. Finally, the percentage of retained drug (C_F) was calculated with respect to the effective drug content (C_0), as follows:

$$C_F = \frac{\mu g\, C_F}{\mu g\, C_0} \cdot 100$$

2.2.10 Antimicrobial tests

The antifungal activity of the gelatin films loaded with Fluconazole was tested *in vitro* against the reference strain *Candida albicans* ATCC 10231 (American Type Culture Collection) and 10 clinical isolates of *Candida* spp. 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). For the analyses, *Candida* strains were cultured on Sabauraud-dextrose agar, the inoculum was prepared in sterile 0.9 % saline solution, and adjusted at 0.5 McFarland, corresponding to 10^6 CFU (colony-forming units)/mL. 1 mL of the fungal suspension was added to 20 mL of Sabauraud-dextrose agar and transferred to an agar plate (Ø = 90 mm). After cooling the inoculated agar at room temperature, disks (Ø = 6 mm) of gelatin -based films were deposited on the surface of the plate. The experiments included the unloaded gelatin films (G_g and G_S70_g) as negative controls, and a paper disk containing 300 μg of Fluconazole as positive control. For this purpose, the drug was solubilized in DMSO at 100 g/L, and 10 μL of a freshly diluted water solution (30 μg/μL) was loaded on the sterile paper disk. After 24 hours of incubation at 37 °C, the antifungal activity was determined by measuring the microbial growth inhibition diameter around the disk.

2.3 Statistical Analysis

One-ways analysis of variance (ANOVA) was employed to assess statistical significance on the mechanical properties, swelling and adhesion tests results. The significance was performed with a Newman-Keuls Multiple Comparison tests. The difference was considered statistically significant with p-value < 0.05.

One-tailed t test was used to compare the two sets of measurements (inhibition zone diameters for fluconazole-loaded gelatin film vs fluconazole control) obtained for each *Candida* spp. The difference was considered statistically significant with p-value < 0.05.

3. RESULTS AND DISCUSSION

The main hurdle facing the efficiency of topical antifungal drugs is their ability to penetrate the skin efficiently. Different formulation strategies have been studied to improve the Fluconazole cutaneous permeation (Salerno et al., 2010; Bachhav et al., 2011; Gupta and Vyas, 2012; El-Housini et al., 2018;), including microemulsion-based hydrogel, micelles and solid lipid nanoparticles. Here, we investigate the characteristics of a novel film forming patch formulation, designed starting from biomaterials as porcine gelatin and snail slime at different volume ratio, and we evaluate its ability of promoting the percutaneous absorption of Fluconazole and its effective antifungal properties.

Gelatin-based films were prepared by mixing the gelatin solution with different amounts of S (Table 1). Films properties were highly modified by S addition: in fact, films became more flexible, stretchable and adhesive on increasing the slime content. Therefore, F was added to the composition containing the highest amount of S, G_S70. All the obtained films were transparent with a color turning more and more yellowish on increasing S content, as it can be observed by comparing the appearance of films G (Figure 1a) and G_S70 (Figure 1b).

F is a white crystalline powder having a columnar form with length between 200-400 µm, as shown in Figure 1e: however, this crystalline form was no longer observed in the film formulation. F completely solubilized in the film forming solution. The appearance of the G_S70_g_F film can be appreciated in Figure 1f: it is very transparent and no presence of the drug crystals could be detected, suggesting the formation of an amorphous solid dispersion after the solvent evaporation. The films surfaces, observed by means of scanning electron microscopy, appeared smooth with no flaw or defect (Fig 1 c,g): even the cross-sections of both G_S70 and G_S70_g_F (Figure 1 d and h, respectively) showed only the layered structure characteristic of gelatin films.

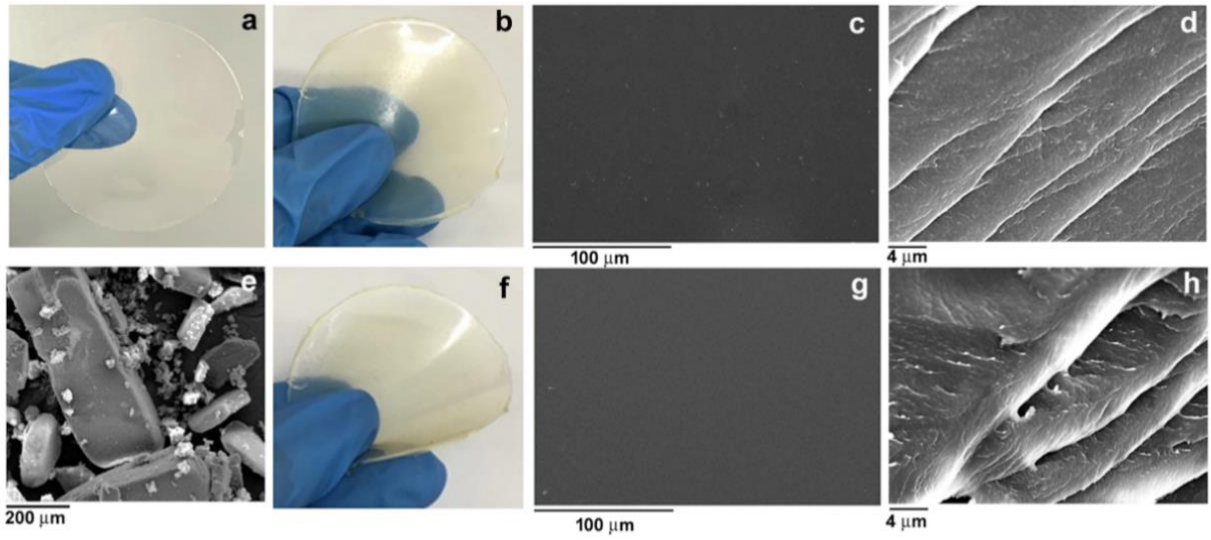

Figure 1: Digital pictures of some selected films: a) G; b) G_S70 and f) G_S70_g_F; Scanning electron micrographs of: e) F crystals; c,d) G_S70, top and cross section, g,h) G_S70_g_F, top and cross section.

3.1 Structural Characterization

Literature reports the existence of at least nine different polymorphic forms of Fluconazole, the main ones being forms I, II, III and a hydrated form (Dash et al, 2001).

The FTIR spectrum collected on F used in this work (Figure 2a) shows a broad band around 3200 cm^{-1} due to hydrogen bonded hydroxyl group stretching vibrations, together with sharp bands between 3100 and 3070

cm^{-1} arising from aromatic C-H stretching vibrations. Moreover, further sharp bands are also detectable. In particular, in the fingerprint region the bands at about 1620, 1600 and 1510 cm^{-1} are associated with the stretching of the C=C and C=N bonds present in triazole, whereas those at 1220 and 1150 cm^{-1} arise from the stretching vibrations of aromatic C-F and from C-O belonging to a tertiary alcohol, respectively. Bands at 1900, 1845 and 1770 cm^{-1} are described as overtone and combination bands consistent with 1,2,4 tri-substitution of phenyl group (Dash et al, 2001). Figure 2b displays the X-ray diffraction pattern recorded from F: the positions and relative intensities of the XRD peaks, as well as those of the infrared absorption bands, are typical of form III polymorph (Dash et al, 2001).

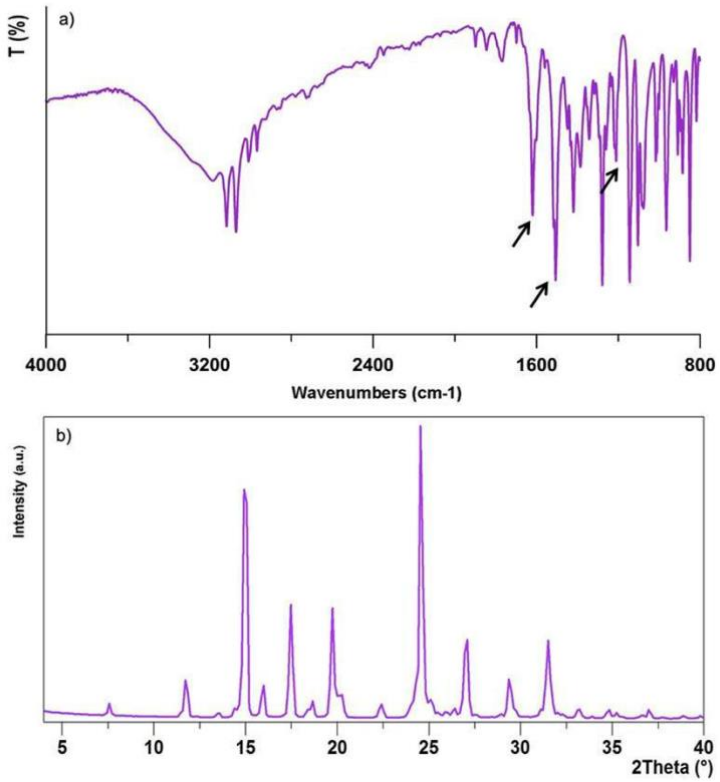

Figure 2: a) FT-IR spectrum and b) X-Ray diffraction pattern of commercial F powder.

FT-IR spectra collected from the composite films are shown in Figure 3. The characteristic bands of the gelatin structure are clearly visible. In particular, the peak at 1653 cm^{-1} can be ascribed to the C=O stretching of amide I, that centered at 1550 cm^{-1} is associated to the NH bending of amide II, while at 1238 cm^{-1} the CN

stretching of amide III can be found (Jiayi et al., 2020). The broad band above 3000 cm^{-1} corresponds to the free and bound hydroxyl and amino groups, whereas the absorption band at about 1330 cm^{-1} is assigned to the wagging of the CH_2 groups of the prolines (Uranga et al., 2016). The main absorption bands of glycerol appear in the 800–1150 cm^{-1} region and are related to the vibrations of C-C and C-O bonds (compare G and G_g spectra) (Guerrero and De la Caba, 2010; Gómez-Siurana et al., 2013). IR spectra of the films containing different amount of snail slime are very similar to each other and show, in addition to the bands belonging to gelatin, two characteristics bands at about 1230 cm^{-1} and at 1719 cm^{-1} ascribable to the presence of allantoin and glycolic acid in the slime extract (Di Filippo et al., 2020).

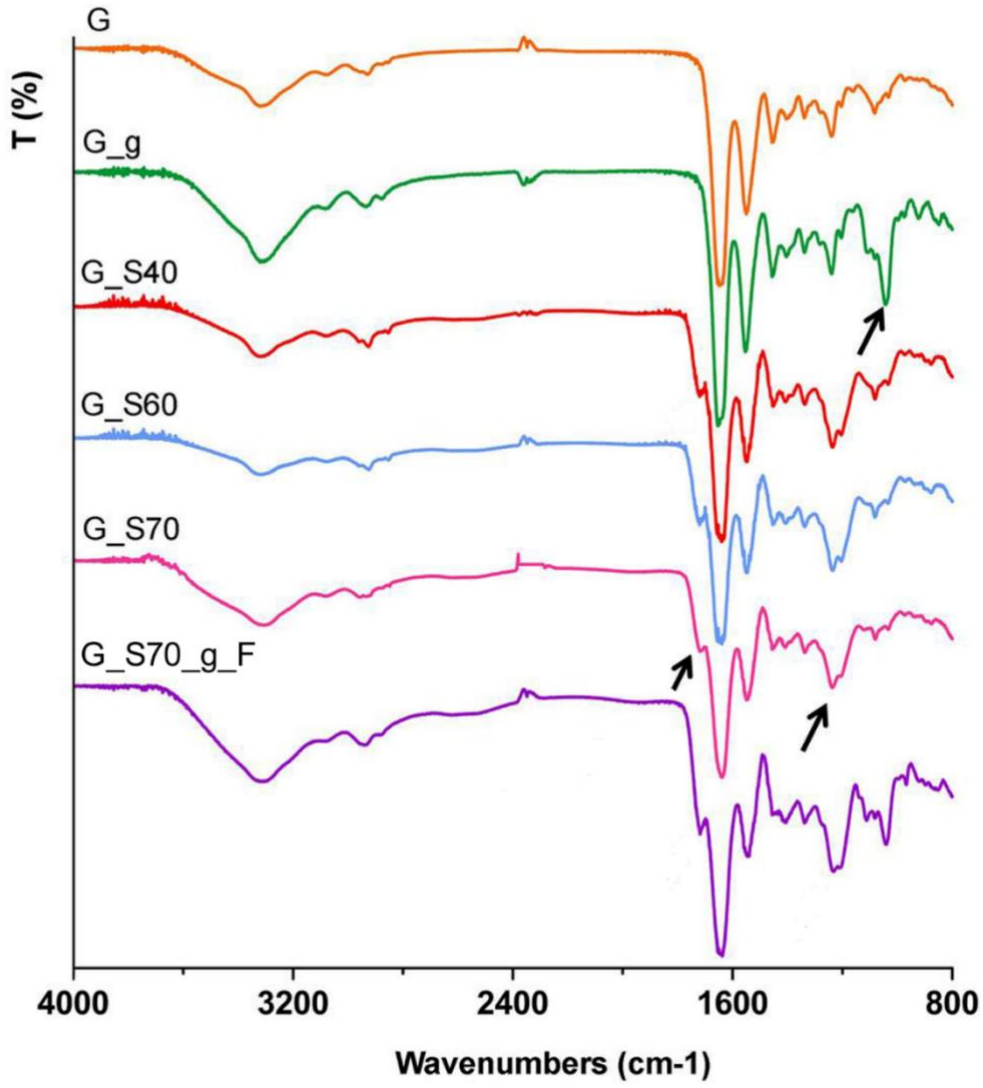

Figure 3: FT-IR spectra collected on gelatin-snail slime -based films. Arrows indicate the most prominent bands attributed to Glycerol and Snail slime.

Surprisingly, addition of F did not modify the IR spectrum and the presence of drug was not revealed by infrared spectroscopy (see G_S70_g_F): this pattern differs from that recorded on G_S70 only for the presence of the bands belonging to glycerol. Infrared spectroscopy and X-rays diffraction were carried out also on a physical mixture of gelatin and F, in the same relative amounts used for the films, in order to verify

the detection limits of these techniques. Obtained spectra reported in Figure S1 and S2, confirmed the presence of polymorph III of Fluconazole. To further support our hypothesis of the formation of an amorphous phase during film casting, no infrared bands belonging to the drug was reported in the literature for Fluconazole and Soluplus films containing less than 30 wt % of the drug (Nowak et al., 2020). In order to confirm the drug solid state into G_S70_g_F film, a structural characterization by means of X-ray diffraction was carried out: diffraction patterns of pristine Fluconazole, G_g, G_S70_g and G_S70_g_F films are compared in Figure 4.

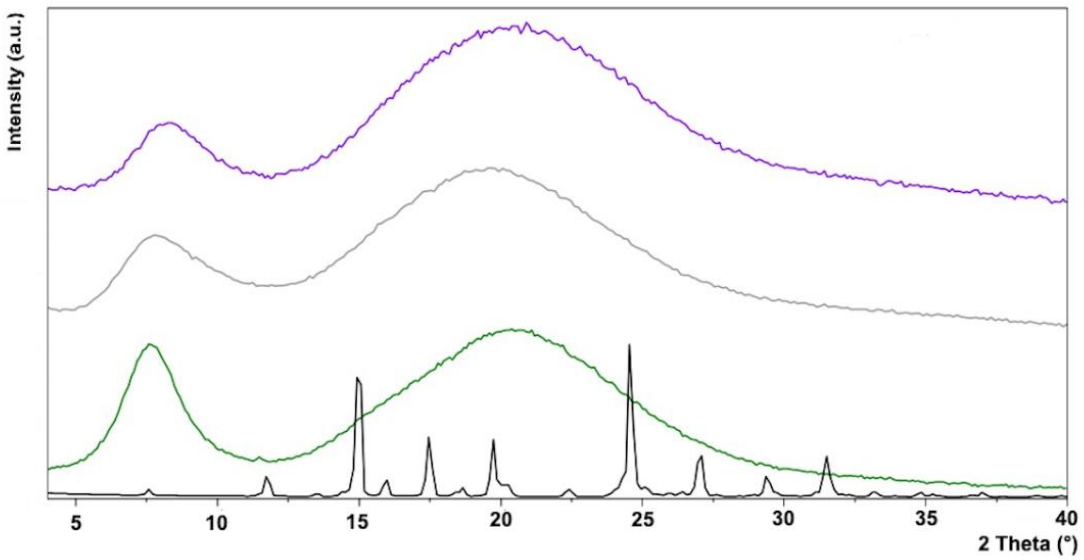

Figure 4: X-Ray diffraction patterns of Fluconazole (black) and of composite films G_g (green), G_S70_g (gray) and G_S70_g_F (purple).

Type A gelatin is characterized by a large reflection at about 8°/2θ and a broad halo centered at 21 °/2θ (Cai et al., 2019). It is known that the peak at ~ 8°/2θ is related to the diameter of the triple helix whereas the halo, corresponding to a periodicity of about 0.45 nm, is related to the distance between adjacent polypeptide strands (Okuyama, 2008; Bigi et al., 2004). Following the addition of snail extract in the film composition (G_S70_g), the reflection centered at about 8°/2θ slightly decreased its intensity, suggesting

that the reconstitution of the triple-helix structure of gelatin was partially destroyed because of its interaction with the natural extract (Wang et al., 2017). This leads to the hypothesis that the presence of S is able to outweigh the polymer-polymer interactions, leading to loss of structural order and, consequently, to the observed reduction in crystallinity, as found for chitosan films (Di Filippo et al., 2020), thus indicating the plasticizing effect of the snail slime. The diffraction pattern of G_S70_g_F did not exhibit the characteristic reflections of the drug, thus supporting the hypothesis of the formation of an amorphous phase after solvent casting. Amorphous forms exhibit high level of supersaturation due to the lack of ordered crystals with high lattice energy state. However, due to their thermodynamically instability, amorphous forms show the tendency of recrystallize, thus negating the solubility enhancement and compromising the therapeutic action. Nowak et al., 2020 reported that solid dispersion of Fluconazole with selected polymers can stabilize the amorphous form of the drug, hindering its recrystallization process during storage. In order to investigate the role of gelatin, glycerol and snail slime on the solid state- transformations of F, some parallel experiments were conducted. We found that the contemporary presence of glycerol and gelatin enhanced the drug dissolution inside the film-forming solution, which appeared clear and without evidence of residual F crystals. However, just after solvent evaporation, films clearly showed the presence of tiny crystals grown inside gelatin layers, as it can be appreciated in Figure 5 a-c. The structural characterization (Figure 5d) revealed that Fluconazole recrystallized just in a few hours as a hydrate polymorph (Dash et al, 2001), the same solid form which was obtained by solvent evaporation of the solution obtained by solubilizing the drug in acidic water (pH 4.5). These findings are very interesting because they highlight the central role of snail slime in stabilizing the amorphous form of the drug. Noteworthy, this effect lasts over time: as a matter of fact, X-rays diffraction and infrared spectroscopy performed on films G_S70_g_F stored for 3 and 6 months (25°C and 50% RH), clearly demonstrated that the drug is still present in the amorphous solid state and the film kept its appearance and transparency (Figure 6).

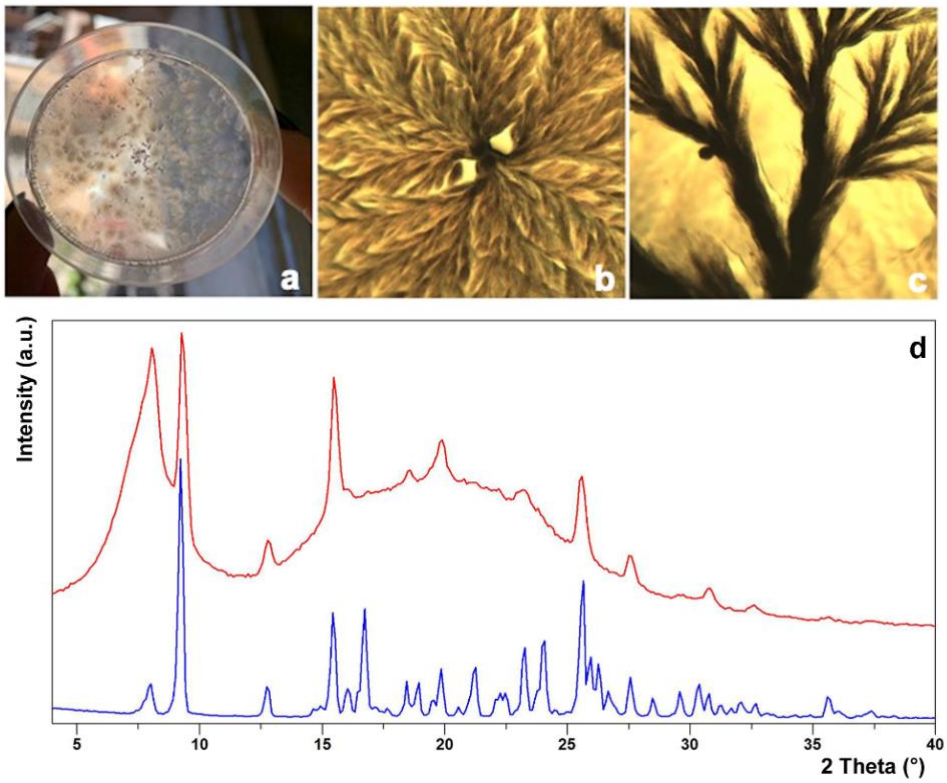

Figure 5: a) Digital image of film G_g_F; b,c) optical microscope images of the crystals grown inside the film; d) X-ray diffraction pattern of G_g_F film (red) and of Fluconazole suspended in distilled water at pH 4.5 (Blu).

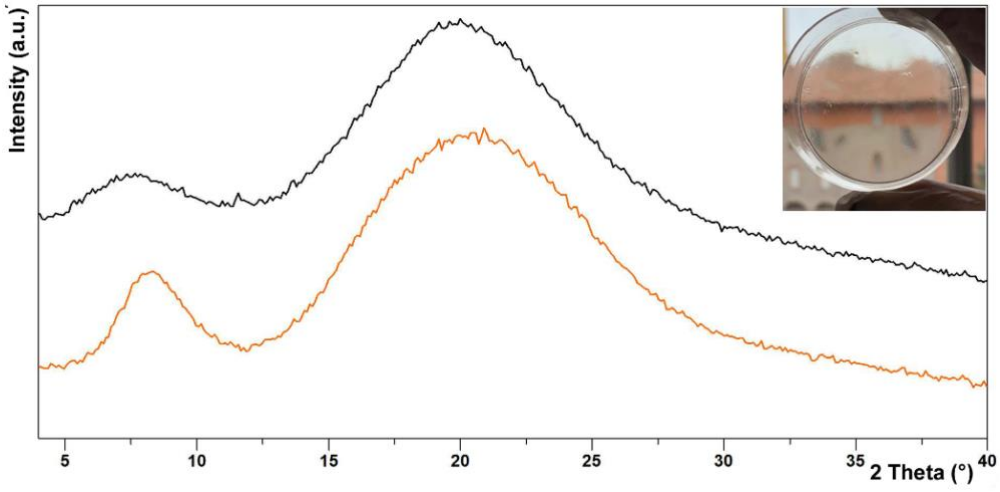

Figure 6: X-ray diffraction pattern of G_S70_g_F after 3 (orange line) and 6 (black line) months of storage. In the insert, the appearance of the film after 6 months of storage.

This phenomenon could be explained by the interactions that gelatin and the Snail Extract (which is a heterogeneous aqueous mixture containing proteins, collagen, allantoin, mucopolysaccharides and glycolic acid) form with F, acting as "amorphous polymer carriers". In fact, according to the literature, amorphous solid dispersions of polymers such as PVP, PVP-VA, HPMC, chitosan and gelatin may increase the solubility and/or the dissolution rate of poorly water soluble drugs, maintaining a sufficient level of supersaturation over storage (Papageorgiou et al., 2008; Karanam et al., 2012; Newman et al., 2012).

3.2 Moisture

The amount of moisture in the film could be crucial as it affects the mechanical strength, adhesive properties and friability of film. TGA analyses were performed on G, G_g, G_S70_g and G_S70_g_F, and the amount of adsorbed water was evaluated from the first loss up to 130°C. G films contained up to 15% (w/w) of water, while those added with Glycerol retained a lower content, 11%, (w/w), and this amount decreased to 8% when glycerol and S were both present into the formulation. Films containing Fluconazole had the lowest amount of adsorbed water: about 2%. It can be inferred that water content has a minor effect on the mechanical properties of films with respect to S addition.

3.3 Tensile tests

The mechanical properties of the films, in terms of maximum stress σ_{max} (MPa), Young's modulus E (MPa) and deformation at break ε_b (%), were evaluated and reported in Figure 7, together with the sample thicknesses.

Snail slime addition produced a dramatic effect on the tensile properties of the films, as clearly evidenced by graphs reported in Figure 7 A-C. As a matter of fact, an impressive decrease of the maximum stress σ_{max} and

of the elastic modulus E was observed for the addition of even the lowest amount of S (G_S40) (aaa: $p < 0.001$ compared to G film) and the values of these parameters decrease on increasing S content (aaa: $p < 0.001$ compared to G film) (see Figure 7A and 7C, respectively). In particular, ongoing from G to G_S70 films, the values of σ and E drop by a factor of about ten, while no significant difference was observed between G_S70, G_S70_g and G_S70_g_F ($p > 0.05$), thus elucidating that the introduction of Fluconazole and/or glycerol did not affect the maximum stress and the elastic modulus of the films. The deformation at break (Figure 7B) slightly decreases up to G_S50 and then increases up to G_S70, reaching a value about 5 times greater than that of G ($^{aaa}p< 0.001$). Therefore, as already observed for chitosan-based films (Di Filippo et al, 2020) it can be inferred that the "plasticizing effect" of Snail Slime increases with its content ($^{bbb}p< 0.001$ for G_S40 compared with G_S70), providing the films with increasing elongation at break. The reason why this trend was not maintained at relatively low S content is not very clear but might be due to different interactions that occur between the polymer and the components of the slime when mixed in different proportions.

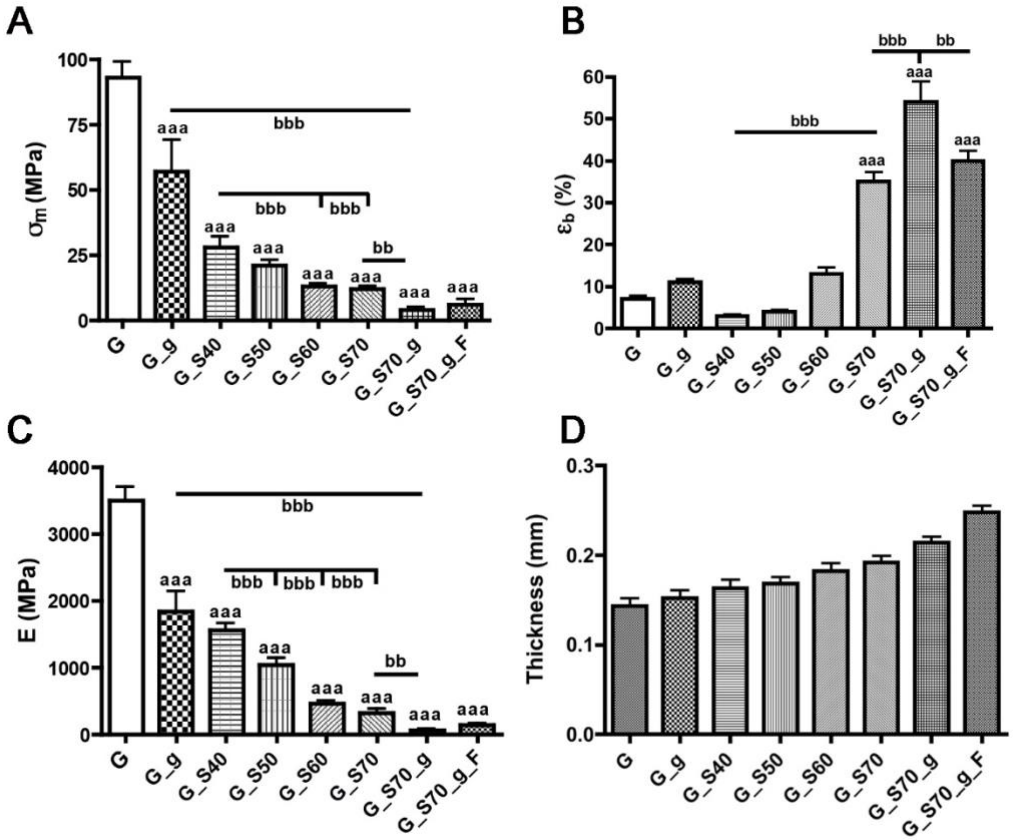

Figure 7: A-C: Mechanical properties of gelatin-based films: A) maximum stress at break (sm, aaap < 0,001 compared to G; bbb p < 0,001 G_g vs G_S70_g, G_S40 vs G_S60 and G_S40 vs G_S70;B) elongation % at break (eb; aaap < 0,001 compared to G; bbb p < 0,001 G_S40 vs G_S70, G_S70 vs G_S70_g and bb p < 0,01 G_S70_g vs G_S70_g_F; C) Elastic modulus (E, aaap < 0,001 compared to G; bbb p < 0,001 G_g vs G_S70_g, G_S40 vs G_S50, G_S40 vs G_S60 and G_S40 vs G_S70; D) Thicknesses of gelatin-based films.

As expected, the presence of glycerol significantly reduced the maximum stress (e.g., the σ_{max} of G_S70 is about 3 times higher than that of G_S70_g, bb: $p < 0.01$) and the elastic modulus, while it enhanced the deformation at break, thus amplifying the effect due to S addition (aaa: $p < 0.001$ for G_S70 and G_S70_g compared to G). Moreover, the presence of glycerol affects the deformation at break (G_S70 compared to G_S70_g, bbb: $p < 0.001$) in agreement with its plasticizer effect. In fact, being glycerol a polyhydroxy-alcohol

with small molecules, it can easily enter the protein matrix to interact with polar amino acids and form hydrogen bonding involving hydroxyl groups, which could alter the forces holding the protein chains together and add free volume between them (Dou et al., 2020). Addition of the drug provoked a small increase of the values of stress at break and elastic modulus together with a slight decrease of the deformation at break, probably due to the lower water content with respect to G_S70_g.

Finally, film's thickness (Figure 7D) increased on increasing the amount of S, according to the larger amount of solid matter: in fact, as previously reported (Di Filippo et al. 2020), snail slime is a mixture of proteins, glycolic acid, allantoin, which represent about a 5% (w/V) of dry matter.

3.4 Swelling behavior

Considering that this film is intended for skin application, swelling of the drug-loaded polymeric film may play an important role on both bioadhesion and controlled drug release (Karki et al., 2016). In fact, hydrophilic polymers with different structures possess a varying degree of swelling based on the relative resistance of matrix network structure to water molecule movement. This may be due to the formation of hydrogen bonding among the polymeric matrix and the other additives: glycerol, S and the drug.

The swelling degree of the films as a function of the snail mucus content, glycerol and F was measured in PB at a pH value of 4.5 and reported in Figure 8.

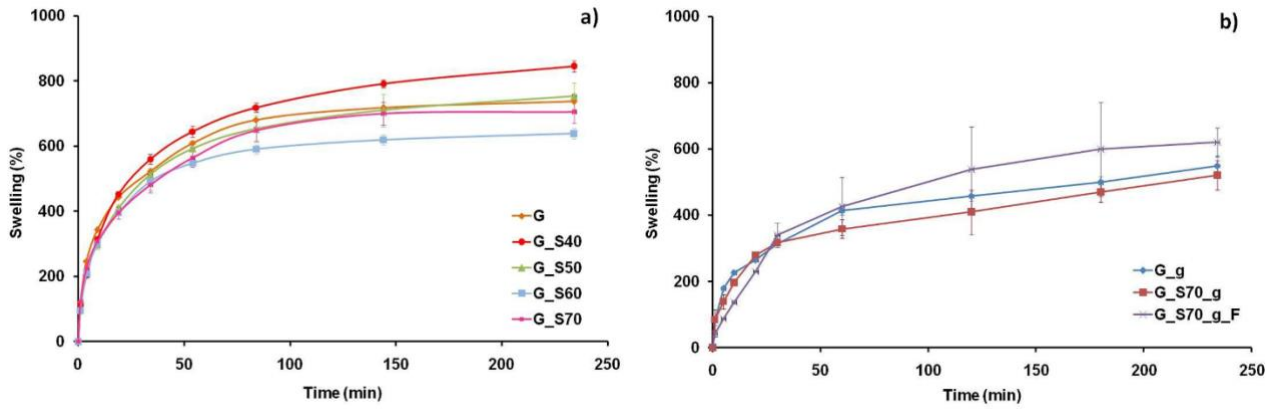

Figure 8: Swelling curves of the gelatin-based films: a) influence of snail slime and b) influence of glycerol and drug.

As shown in Figure 8a, swelling of all the samples gradually increases up to about 150 minutes, when it reaches an almost constant plateau. The presence of the snail slime does not seem to significantly affect the swelling properties of the films ($p>0.05$) and a clear trend as a function of S content could not be found. All the samples containing glycerol showed (Fig. 8b) a marked reduction of the swelling degree compared to those without glycerol, indicating the formation of strong interactions between glycerol and gelatin. The addition of S at 70% to G _g (Figure 8b), as well as the addition of Fluconazole, does not significantly modify the swelling ability with respect to the G_g film.

3.5 Skin adhesion studies

Skin adhesion is one of the most important functional property for a skin drug delivery system.

Actually, gelatin and snail lime -based films (G_S70 and G_S70_g_F) reported in Figure 1 b and f are slightly adhesive to the touch, but they behave like patch only when their surface in contact with the skin is wetted. Therefore, such films, once removed from the primary packaging, might be used simply by wetting the skin with water (few droplets) and then applying the film on the wet skin with light pressure to make it adhere well to the edges. Moreover, the capability of the films to remain attached to the skin for several hours was tested up to 8 hours: as an example, Figure 9b shows a digital picture of G_S70_g film attached to the skin of a hand by means of few drops of water, immediately after adhesion and 8 hours later. Film is highly performant and keeps its adhesive force over time.

The results of the adhesion strength measurements, expressed as the Force (N) necessary to detach the film adhered to the skin, are shown in Figure 9a.

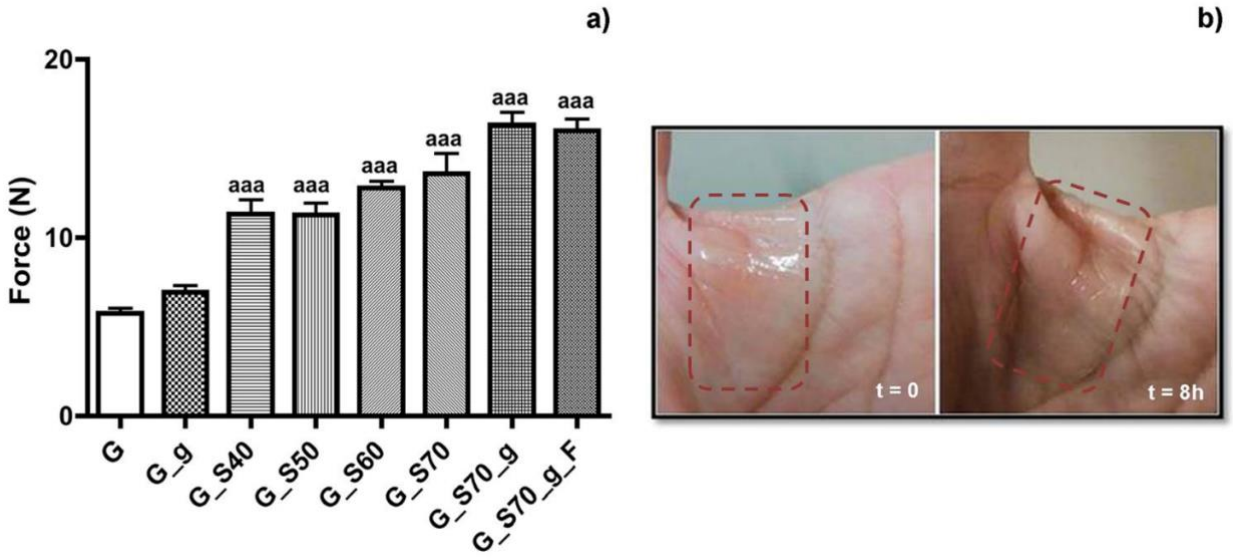

Figure 9: a) Adhesive strength values of the films expressed as Force (N) ± SD (aaa: p< 0.001 all samples compared to G; b: p< 0.05 for G_S70 vs G_S70_g); b) Digital picture of G_S70_g film adhered to the skin of a human hand immediately after adhesion and after 8 hours.

The trend of the histogram suggests that increasing quantities of S in the composition of the films cause an increase in their adhesive capacity. The film with the best adhesive capacity is the one with the higher amount of S, G_S70, with a value more than 2 times higher than G (aa: p< 0.001).

As highlighted by the histogram, also glycerol was able to increase the adhesive capacity of the samples containing S: in fact, 17% more strength was required to detach the films G_S70_g from the skin, when compared to G_S70 (p<0.05).

Interestingly, even if the adhesion capacity of the G_g film is higher than that of the G film, it is lower compared to G_S40, suggesting that snail slime played a major role on promoting adhesion. On the other hand, the presence of the drug did not significantly affect the adhesive ability of the film.

In general, all the obtained films showed good detachment forces from the skin, guaranteeing their adhesion to the skin without the addition of glues, like pressure sensitive adhesives generally used in patch development. For example, methyl methacrylate copolymers (Eudragits) combined with a large amount of plasticizers are generally used to design the pressure-sensitive adhesives which have to assure the contact with the skin, as well as drug penetration into the skin (Cilurzo et al., 2014).

Therefore, the adhesive effect of this monolayer film forming patch may be attributed to the peculiar mixture of proteins and polysaccharides of the snail slime, similarly to the adhesive property of the mucus trail (Newar and Ghatak, 2015).

3.6 In vitro permeation studies

To assess the permeation profile of Fluconazole from the film, the effective amount of F loaded into the film was first assessed. The experimental drug content was 4.53 % ± 0.07 w/w (corresponding to 1.84±0.05 mg/cm^2), very close to the theoretical one (4.76% w/w) suggesting that the preparative methods allowed to obtain a homogenous drug distribution within the formulation.

Permeation results obtained from G_S70_g_F film were compared with a control solution containing a drug concentration of 4.14±0.02 mg/ml. A certain amount of ethanol (20% m/V) was added in the control solution to prevent F precipitation, since the drug saturated solubility in pH 5.5 phosphate buffer solution at 25°C was 5.05±0.05 mg/ml, very close the reported water solubility (4.37 mg/ml). The drug solubility increased up to 16.43±0.85 mg/ml in the control solution. The same solution was used in the receiver compartment to ensure sink conditions in the permeation experiments and the drug concentration did not exceeded 20% of the saturated solubility (20.07±1.09 mg/ml).

Analyzing the permeation results, the amount of permeated drug from the solid formulation was superimposable to that of the drug in solution (Figure 10). In particular, in both systems the cumulative amount of F permeated through the skin was about 60 μg/cm^2 (corresponding to 5 % of F) after two hours, 200 μg/cm^2 after 4 hours and approximately 500 μg/cm^2 in 8 hours. After 24 hours the drug permeation reached 1000 μg/cm^2 corresponding to 60% of F loaded into the film. These results evidence that the film formulation enhanced the drug permeation into the skin layer displaying a similar behavior to the control solution containing 20% of ethanol w/V, which is known for its penetration enhancement property (Alberti et al., 2001). A possible explanation of the film behavior can be related to the different thermodynamic activity of the examined samples. The drug loaded film formulation (G_S70_g_F) is an example of a supersaturating drug delivery system. As reported in the section 3.1 "Structural characterization", the obtained film is an amorphous solid dispersion, which demonstrated a supersaturation stability for 6 months, unlike the films without snail slime, in which F quickly recrystallized. On the other hand, the control solution contains the drug at a concentration lower than its saturated solubility (16.43±0.85 mg/ml). Thus, for the control solution, the permeation profile of F across the skin is promoted by the enhancer, mainly through a lipid extraction mechanism (Gupta et al., 2020), rather than by the thermodynamic activity of the subsaturated drug solution. In contrast to chemical penetration enhancers, like ethanol, supersaturation has proved to increase skin penetration without alteration of the stratum corneum (Moser et al., 2001). The increased thermodynamic activity associated to the supersaturation degree of the film formulation might be a valid explanation of the permeation behavior of the drug. In fact, the film maintained a sufficient level of supersaturation over the analysis time, ensuring complete dissolution and good permeation. Thus, the hybrid film containing the biopolymer and the snail slime might represent an effective drug delivery system to the skin.

Finally, the amount of drug accumulated in viable skin measured at the end of the experiments were 9.6 ± 0.6% (corresponding to 176.0± 11.1 μg/cm^2) and 6.8 ± 1.9% (corresponding to 140.8 ± 39.1 μg/cm^2) for film and solution, respectively, indicating a certain drug accumulation into the skin.

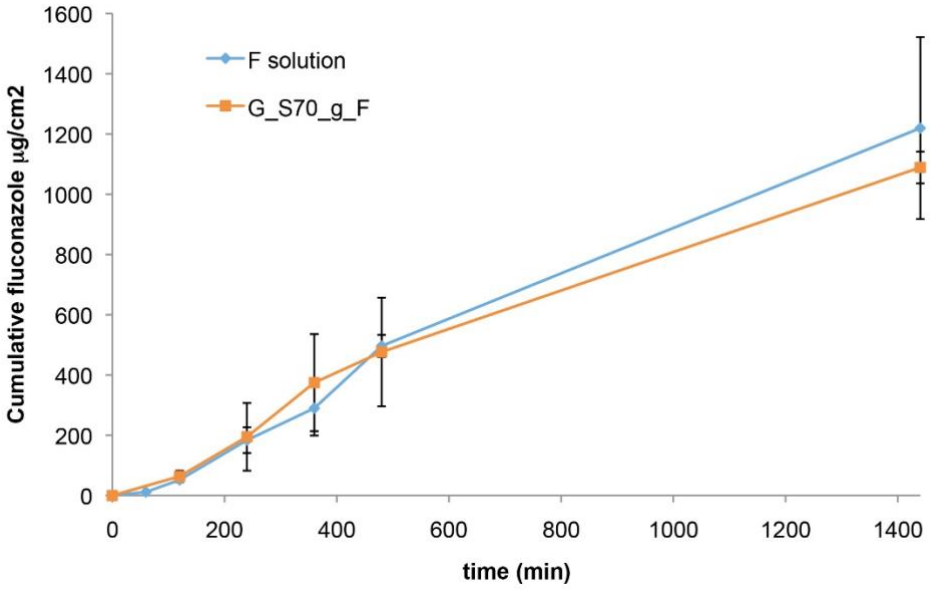

Figure 10: Cumulative fluconazole concentration permeated through pig ear skin: G_S70_g_F (orange) and F solution (blue) as a control (0,45% w/V solution of F in PB supplemented with 20% w/w EtOH corresponding to the same amount of F loaded into the film).

3.7 Antimicrobial tests

The Fluconazole-loaded gelatin films were tested *in vitro* for the evaluation of the antifungal activity against the reference strain *C. albicans* ATCC 10231 and 10 clinical isolates of *Candida* spp. As negative control, unloaded gelatin films (based on neat gelatin and on the mixture gelatin and snail slime) and Fluconazole paper disks as positive control were included in each experiment. Table 2 reports the diameters of the inhibition growth zones, measured around the tested disks and the Fluconazole control (300 μg). As

expected, the unloaded films did not display any antifungal activity. No statistically significant differences between G_S70 _g_F and the positive control were observed on the two sets of measurements. These results are in agreement with the permeation studies and are attributable to the supersaturating status of the novel film formulation. Therefore, Fluconazole-loaded gelatin and snail slime-based films, containing 320-360 µg of drug, proved to be effective towards *Candida* spp. as well as the positive control, indicating that the drug loaded into the film quickly dissolved freely diffused through the agar maintaining its high potency and with the same activity of the drug solution.

Table 2. Measurements of the inhibition zone diameter (mm) detected for unloaded and Fluconazole-loaded gelatin films. Values are mean and standard deviation of two independent experiments performed on the reference strain and on two isolates for each *Candida* spp.

	G_g	**G_S70_g**	**G_S70_g_F**	**F**
C. albicans ATCC 10231	N.A.*	N.A.*	26,5 ± 0,6	25,6 ± 0,6
C. albicans (n = 2)	N.A.*	N.A.*	23,5 ± 4,7	23,6 ± 2,1
C. glabrata (n = 2)	N.A.*	N.A.*	24,5 ± 1,3	25,0 ± 0,9
C. parapsilosis (n = 2)	N.A.*	N.A.*	23,5 ± 0,7	22,7 ± 1,2
C. tropicalis (n = 2)	N.A.*	N.A.*	22,0 ± 1,8	22,6 ± 1,8
C. krusei (n = 2)	N.A.*	N.A.*	21,5 ± 2,4	23,1 ± 1,6

*N.A. not appearing

4. CONCLUSIONS

A new film-forming patch based on gelatin and snail slime for the local delivery of the model drug, Fluconazole, was successfully developed.

Film properties significantly depend on the amount of snail slime: in particular, formulations with the higher contents of S are more flexible and stretchable and the presence of the slime provides the films with the desirable adhesive properties towards the skin. Films are easy to handle and, once applied to the skin, the adhesion is provided for more than 8 hours. The film containing the largest volume of extract was selected as potential delivery platform of Fluconazole. The presence of snail slime promotes drug solubilization into the film forming solution and hinders Fluconazole recrystallization inside the film. Furthermore, we demonstrated that snail mucus is crucial in stabilizing the amorphous form over the time, until 6 months of storage. Patches containing Fluconazole demonstrate an effective antifungal activity against all the tested *Candida strains* and the drug permeation across the skin.

In conclusion, the novel patch-formulation based on natural and biodegradable materials developed in this study is able to adhere to the wet skin, promoting Fluconazole permeation, and can be easily washed off, avoiding waste, or thrown into compost.

Acknowledgments

The authors thank “I Poderi” farm (Montemerano, Italy) for providing them with snail slime extract from Helix Aspersa Muller with the MullerOne extraction method.

The authors thank “Bottega Ranocchi 1972” Butcher's shop (Bologna, Italy) for providing them with the pork tissue.

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