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Júlia Conte

**DESENVOLVIMENTO DE FILMES VAGINAIS PARA O TRATAMENTO DE
CANDIDÍASE VULVOVAGINAL RESISTENTE**

Florianópolis

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Júlia Conte

**DESENVOLVIMENTO DE FILMES VAGINAIS PARA O TRATAMENTO DE
CANDIDÍASE VULVOVAGINAL RESISTENTE**

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Orientador: Prof. Dr. Thiago Caon
Coorientador: Prof. Dr. Alexandre Luis Parize

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O presente trabalho em nível de mestrado foi avaliado e aprovado em 01/09/2023 por banca examinadora composta pelos seguintes membros:



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RESUMO

A candidíase vulvovaginal é comumente tratada com antifúngicos azólicos e poliênicos por via oral ou local. A administração oral destes fármacos pode ser associada com efeitos colaterais sistêmicos tais como hepatotoxicidade. A administração vaginal é vantajosa já que evita o metabolismo de primeira passagem e permite concentrar localmente o fármaco. Formas farmacêuticas semissólidas tais como cremes e pomadas são geralmente utilizadas. Apesar da fácil espalhabilidade, estas formulações possuem baixa retenção na mucosa, menor controle da dose aplicada e maior susceptibilidade à contaminação microbiana. Por esta razão, formas farmacêuticas sólidas têm sido priorizadas. Na primeira etapa deste trabalho, realizou-se uma revisão da literatura a fim de identificar aspectos tecnológicos de formas farmacêuticas sólidas inovadoras (filmes, esponjas, nanofibras e anéis poliméricos) para a referida patologia. Vantagens e desvantagens destas formulações, métodos de preparo, perfis de liberação e resultados de atividade frente a cepas causadoras da infecção foram abordados em detalhes. Ainda, apresentaram-se aspectos envolvendo a patogênese da candidíase, tratamento convencional atual e o uso da mucosa vaginal como rota alternativa à administração de fármacos. Filmes poliméricos têm tido destaque devido a versatilidade de técnicas e materiais que podem ser empregados na produção, o que motivou a seleção desta forma farmacêutica para a etapa experimental. Nesta ocasião, filmes biopoliméricos de quitosana ou *blends* deste polímero com pectina e acetato succinato de hidroxipropilmetilcelulose (HPMCAS) foram desenvolvidos pelo método de evaporação do solvente e então caracterizados. Fluconazol foi selecionado como antifúngico e o timol, composto majoritário do óleo essencial de tomilho, como promotor de absorção. Análises térmicas e espectroscópicas indicaram compatibilidade química entre os componentes do filme. Ambos fluconazol e polímeros encontraram-se em uma forma amorfa após a secagem. Filmes de quitosana e HPMCAS mostraram maior plasticidade e flexibilidade, explicando a maior taxa de intumescimento em fluido vaginal. A combinação de ácido cítrico e ácido lático, que foi utilizada para a solubilização da quitosana, desempenhou um papel como plastificante. Além de melhorar as propriedades mecânicas dos filmes, a combinação de biopolímeros tornou os filmes mais hidrofílicos, o que facilitaria o acesso de fluidos corpóreos. Todas as formulações proporcionaram altas taxas de permeabilidade do fluconazol em mucosa vaginal suína, o que pode ser atribuído à capacidade da quitosana em afrouxar as junções aderentes encontradas entre as células epiteliais. O timol mostrou ser um promotor de absorção eficaz e a sua incorporação nos filmes também foi vantajosa para melhorar a atividade antifúngica do fluconazol, diminuindo a dose efetiva em 50% frente a *C. glabrata* (cepa resistente e de grande relevância clínica). De posse destes resultados, é possível afirmar que os filmes a base de biopolímeros representam uma alternativa promissora para o tratamento dos casos resistentes desta patologia, motivando a realização de ensaios clínicos. Os filmes de quitosana/HPMCAS deveriam ser priorizados nesses novos estudos, pois mostraram melhores propriedades mecânicas e maior taxa de intumescimento, o que levaria a uma liberação sustentada do fármaco.

Palavras chaves: candidíase; administração vaginal; filmes poliméricos; fluconazol; timol.

ABSTRACT

Vulvovaginal candidiasis is commonly treated with oral or local azole and polyene antifungals. Oral administration of these drugs can be associated with various systemic side effects such as hepatotoxicity. In this context, vaginal administration is advantageous as it avoids first-pass metabolism and allows a more local distribution of the drug. Semi-solid formulations such as creams and ointments have been often used. Although they are easy to spread, low retention in the mucosa, less control the applied dose and greater susceptibility to microbial contamination can be found. For this reason, solid dosage forms have been prioritized. In the first stage of this study, a literature review was carried out to identify technological aspects of innovative solid dosage forms (polymer films, sponges, nanofibers and vaginal rings) for the aforementioned pathology. Advantages and disadvantages of these formulations, preparation methods, release profiles and activity results against the strains causing the infection were discussed in detail. Moreover, aspects involving the pathogenesis of candidiasis, current conventional treatment and the use of the vaginal mucosa as an alternative route for drug administration were presented. Polymer films have received attention due to the versatility of techniques and materials that can be used in the preparation, which motivated the selection of this pharmaceutical form for the experimental stage. On this occasion, chitosan films or blends of this polymer with pectin and hydroxypropylmethylcellulose acetate succinate (HPMCAS) were developed by the solvent evaporation technique and then characterized. Fluconazole was selected as an antifungal and thymol, the major compound of thyme essential oil, as an absorption enhancer. Thermal and spectroscopic analyzes indicated chemical compatibility between the film constituents. Fluconazole and polymers were found in an amorphous form after drying. Chitosan and HPMCAS films showed greater plasticity and flexibility, explaining the higher swelling rate in presence of vaginal fluid. The combination of citric acid and lactic acid, which was used for chitosan solubilization, played a role as a plasticizer. In addition to improving the mechanical properties of the films, the combination of biopolymers made the films more hydrophilic, which would facilitate the access of body fluids. All formulations provided high permeability rates for fluconazole through the porcine vaginal mucosa, which can be attributed to the ability of chitosan to loosen adherent junctions between epithelial cells. Thymol proved to be an effective absorption enhancer and its incorporation into the films was also advantageous to improve the antifungal activity of fluconazole, decreasing the effective dose by 50% against *C. glabrata* (resistant strain of great clinical relevance). With these results in hand, it is possible to state that films based on biopolymers represent a promising alternative for the treatment of resistant cases of this pathology, encouraging clinical trials. Chitosan/HPMCAS films should be prioritized in these new studies as they demonstrated better mechanical properties and a higher swelling rate, which would contribute to a sustained drug release.

Keywords: candidiasis; vaginal administration; polymeric films; fluconazole; thymol.

LISTA DE FIGURAS

Capítulo I – Revisão Bibliográfica

Figura 1. Estrutura química do fluconazol	255
Figura 2. Estrutura química do timol.....	277
Figura 3. Técnica de evaporação de solvente	311
Figura 4. Estrutura química da quitosana desacetilada	322
Figura 5. Estrutura química do ácido poligalacturônico	333
Figura 6. Estrutura química do acetato succinato de hidroxipropilmetilcelulose	344

Capítulo II – Artigo publicado

Figure 1. Schematic representation of adhesion, invasion, and infection by <i>Candida</i> sp. by (a) endocytosis and (b) active penetration.	544
Figure 2. Different intravaginal ring configurations: (a) reservoir-type; (b) sandwich; (c) matrix-type ring and (d) pod-type ring design.	60
Figure 3. Cumulative release profile of (a) reservoir-type ring; (b) sandwich; (c) matrix-type and (d) pod-type ring design.....	60
Figure 4. Schematic representation of electrospinning process.	71

Capítulo III – Artigo a ser submetido para publicação

Figure 1. Thermogravimetric analysis of pure constituents (A and B) and polymer films (C, D, E, F, G and H).....	106
Figure 2. DSC thermograms of acids (A), fluconazole and thymol (C), polymers (E), and films (B, D and F).....	108
Figure 3. FT-IR spectra of raw materials (A, C and E) and polymer films (B, D and F).....	111
Figure 4. X-ray diffraction patterns of CH-based films (FI – FIX).....	112
Figure 5. Stress-strain curves of chitosan films (FI - FIII), chitosan/pectin films (FIV-FVI) and chitosan/HPMCAS films (FVII-FIX).....	114
Figure 6. Swelling behavior of developed films from FI to FIX.....	117
Figure 7. SEM imagens of surface and cross-sections of films.....	118
Figure 8. Permeability profile of fluconazole from polymeric films through the porcine vaginal mucosa.....	120

Figure 9. ATR-FTIR spectra of porcine vaginal mucosa after treatment with PBS (blank/control) and FII, FIII, FVI and FIX.....	122
Figure 10. Anti- <i>Candida</i> activity of chitosan-based films loaded with fluconazole (FLU) and/or thymol (THY) against <i>C. glabrata</i> strain. (A) CH-film with FLU (512 µg/disk); (B) CH-film with THY (2,048 µg/disk); (C) CH-film with FLU and THY (512 and 2,048 µg/disk, respectively) and (D) CH-film with FLU and THY (256 and 1,024 µg/disk, respectively).....	123
Supplementary Figure S1. X-ray diffraction pattern of crystalline fluconazole.....	137
Supplementary Figure S2. Fluconazole retained in porcine vaginal mucosa after 7h.....	137

LISTA DE TABELAS E QUADROS

Capítulo I – Revisão Bibliográfica

Quadro 1. Tratamento da candidíase vaginal preconizado pelo Ministério da Saúde do Brasil.....	21
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Capítulo II – Artigo publicado

Table I. Novel solid drug delivery systems developed for antifungal agents in vulvovaginal candidiasis.....	75
Table II. Advantages and disadvantages of advanced solid formulations for vulvovaginal candidiasis.....	78

Capítulo III – Artigo a ser submetido para publicação

Table 1. Composition of biopolymer films for vaginal infections.....	97
Table 2. Thickness values of developed films.....	103
Table 3. Tensile data of developed film.....	115
Table 4. Contact angle of developed films.....	116
Table 5. Permeability parameters of fluconazole from control solutions and film formulations.....	120
Supplementary Table S1. Thermal events in TG analysis of pure constituents and polymer films.....	135
Supplementary Table S2. Thermal characteristics of the film and pure constituents obtained from DSC analyses.....	136

LISTA DE ABREVIATURAS E SIGLAS

CDR - *Candida drug resistance*

CIM - Concentrações inibitórias mínimas

CVV - Candidíase vulvovaginal

CVVR - Candidíase vulvovaginal recorrente

DRX - Difração de raios-X

DSC - Calorimetria diferencial exploratória / *Differential scanning calorimetry*

EPS - *Extracellular polymeric substances*

FF- Formas farmacêuticas

FLU - Fluconazol

FT-IR - Espectroscopia na região do infravermelho com transformada de Fourier / *Fourier-transform infrared spectroscopy*

GM - Grau de metoxilação

HPMC - Hidroxipropilmetilcelulose

HPMCAS - Acetato succinato de hidroxipropilmetilcelulose

MEV - Microscopia eletrônica de varredura

TGA - Análise termogravimétrica / *Thermogravimetric analysis*

SUMÁRIO

1 INTRODUÇÃO.....	15
2 OBJETIVOS.....	18
2.1 OBJETIVO GERAL.....	18
2.2 OBJETIVOS ESPECÍFICOS	18
3 CAPÍTULO I – REVISÃO BIBLIOGRÁFICA.....	19
3.1 CANDIDÍASE VULVOVAGINAL	19
3.1.1 Sinais e sintomas	19
3.1.2 Tratamento.....	20
3.1.2.1 Mecanismos de resistência	22
3.2 ADMINISTRAÇÃO VAGINAL DE FÁRMACOS	23
3.2.1 Anatomia	23
3.2.2 Rotas de permeabilidade.....	24
3.3 FLUCONAZOL	24
3.3.1 Características físico-químicas	24
3.3.2 Mecanismo de ação	25
3.3.3 Farmacocinética.....	26
3.3.4 Efeitos adversos.....	26
3.4 TIMOL	27
3.4.1 Características físico-químicas	27
3.4.2 Promoção da absorção em mucosa vaginal	27
3.4.3 Atividades biológicas	28
3.5 FILMES POLIMÉRICOS	29
3.5.1 Métodos de preparo	30
3.5.2 Polímeros	31
3.5.2.1 Quitosana	31
3.5.2.2 Pectina	32

3.5.2.3 Acetato succinato de hidroxipropilmetilcelulose	33
3.5.3 Caracterização físico-química	35
3.5.3.1 Força de tração e percentagem de alongamento	35
3.5.3.2 Análise térmica	35
3.5.3.3 Espectroscopia na região do infravermelho (FT-IR)	36
3.5.3.4 Intumescimento e ângulo de contato	36
3.5.3.5 Morfologia	37
3.5.3.6 Mucoadesão e tempo de residência in vitro	37
4 CAPÍTULO II – ARTIGO PUBLICADO	48
4.1 ARTIGO	49
5 CAPÍTULO III – ARTIGO A SER SUBMETIDO PARA PUBLICAÇÃO	91
5.1 ARTIGO	92
6 DISCUSSÃO GERAL	138
7 CONSIDERAÇÕES FINAIS	144

1 INTRODUÇÃO

A candidíase vulvovaginal (CVV) é a segunda infecção vaginal mais frequente entre as mulheres. Aproximadamente 75% delas apresentam pelo menos um episódio desta patologia durante a vida e cerca de 20% até quatro episódios por ano. Este último caso caracteriza a conhecida *candidíase vulvovaginal recorrente* (CVVR). De acordo com estudos epidemiológicos, há uma incidência de 138 milhões de casos por ano (DENNING *et al.*, 2018).

Os fungos responsáveis pela infecção (do gênero *Candida*) integram a microbiota do tecido vaginal e proliferam de maneira descontrolada particularmente em mulheres que se encontram em um estado debilitado ou imunocomprometido. Com isto, os microrganismos aderem e invadem a mucosa vaginal, causando inflamação, coceira, edema, dor e a formação de placas brancas, observando-se um corrimento branco e grumoso (BRASIL, 2022; PAPPAS, 2006).

O tratamento da CVV é realizado tanto pela via oral quanto local, principalmente pelos fármacos antifúngicos azólicos (miconazol, fluconazol, itraconazol), mas também por polienos. Nistatina e anfotericina B são os principais representantes desta última classe. Ambas as classes de medicamentos agem na parede fúngica inibindo a síntese do ergosterol (componente essencial da estrutura) ou alterando a permeabilidade da parede do patógeno (COSTA *et al.*, 2003). O fluconazol (FLU), fármaco triazólico utilizado na terapia da CVV, é metabolizado principalmente no fígado. Isto leva a efeitos hepatotóxicos indesejáveis bem como ocorrência de interações medicamentosas. Efeitos colaterais tais como dores de cabeça, cardiomiopatia e distúrbios gastrointestinais podem também ser observados (NSENKA & BONGOMIN, 2022). Ainda, o tratamento oral é contraindicado para gestantes, grupo que possui uma alta reincidência da CVV (MØLGAARD-NIELSEN *et al.*, 2016).

Em geral, o tratamento das pacientes inicia sem o diagnóstico do agente etiológico, contribuindo para o uso indiscriminado de medicamentos. Há também o uso profilático e, em casos de resistência, os tratamentos são de longa duração. Efeitos adversos contribuem para reduzir a taxa de adesão das pacientes. Todas estas situações favorecem o aparecimento de cepas resistentes da *Candida*, sendo necessário um aumento da dose, o que pode aumentar a ocorrência de efeitos adversos, ou então a associação de medicamentos (SOARES *et al.*, 2018). Além dos mecanismos de resistência específicos das cepas de *Candida*, como superexpressão de genes de bomba

de efluxo, biofilmes podem ser formados. Tratam-se de estruturas tridimensionais e complexas que restringem a entrada de células de defesa e fármacos (PALMEIRA-DE-OLIVEIRA *et al.*, 2015).

Frente a isto, este estudo teve como foco o desenvolvimento de uma formulação tópica para superar estas limitações. Esta rota de administração traz como vantagens um maior controle da liberação (menor interferência de fatores fisiológicos que a rota oral) e disponibilidade dos ativos no local de ação, incremento de eficácia, maior adesão da paciente ao tratamento bem como facilidade de aplicação. Trata-se de via não-invasiva que evita os efeitos colaterais sistêmicos associados a administração oral (MACHADO *et al.*, 2015).

Caso o fármaco apresente baixa permeabilidade na mucosa vaginal, o uso de promotores de absorção pode ser considerado. Estes agentes causam uma desordem temporária em sítios lipídicos e/ou proteicos do tecido, aumentando a penetração do fármaco. Terpenos tem já sido utilizados para este propósito. O timol, um monoterpene aromático, além de atuar como promotor de permeação, tem demonstrado atividade antifúngica frente a biofilmes de *Candida*, agindo sinergicamente com o fluconazol (AMARAL *et al.*, 2020; JAFRI & AHMAD, 2020). Assim, apresenta-se como um candidato promissor para a incorporação em formulações destinadas ao tratamento da candidíase.

Pomadas, géis, cremes, supositórios e óvulos têm sido as formas farmacêuticas (FF) tradicionalmente utilizadas para a administração vaginal de fármacos. Ainda que as FF semissólidas possam ser facilmente distribuídas na cavidade vaginal, apresentam como desvantagens o extravasamento do canal vaginal, incômodo à paciente, alta frequência de administração, baixa adesão ao tratamento, além de falta de uniformidade da dose, baixa biodisponibilidade e estabilidade, bem como maior suscetibilidade à contaminação bacteriana devido ao alto teor de água (JOHAL *et al.*, 2016).

Por conta disto, FF sólidas têm sido priorizadas, pois permitem modular a liberação dos fármacos, apresentam maior estabilidade à degradação e oxidação, têm baixo teor de água e proporcionam maior conforto à paciente já que não ocorre extravasamento pelo canal vaginal. Nos últimos anos, os anéis vaginais, filmes, esponjas e nanofibras poliméricas têm sido extensivamente pesquisados e desenvolvidos para esta finalidade (CALVO *et al.*, 2019; NEMATPOUR *et al.*, 2020; SHAKER *et al.*, 2018), o que foi abordado na primeira etapa desta dissertação (sistematizada na forma de um artigo de revisão). Filmes poliméricos foram as formas farmacêuticas sólidas

mais estudadas, o que pode ter relação com a simplicidade no preparo, baixo custo, bem como possibilidade de se combinarem diferentes polímeros ou desenvolverem sistemas multicamadas a fim de alcançar perfis de liberação específicos (REAL *et al.*, 2013).

Como uma segunda etapa desta dissertação, filmes poliméricos contendo fluconazol e timol foram desenvolvidos a fim de tratar candidíase vulvovaginal “resistente”. Os biopolímeros quitosana, pectina e acetato succinato de hidroxipropilmetilcelulose (HPMCAS) foram selecionados devido à alta biocompatibilidade e propriedades formadoras de filme. A HPMCAS é um polímero semissintético derivado da celulose, de natureza não-iônica, que é capaz de se dissolver na presença de fluido vaginal (NOTARIO-PÉREZ *et al.*, 2019). A pectina é um polissacarídeo formado por monômeros de ácido galacturônico, que são unidos entre si por ligações glicosídicas. Destaca-se por ser biodegradável e, quando combinado à quitosana, forma ligações cruzadas que aumentam o tempo de residência da formulação no local de ação (LAZARIDOU & BILIADERIS, 2020; MISHRA *et al.*, 2017). Após o preparo, os filmes foram caracterizados quanto as propriedades térmicas, morfológicas, espectroscópicas, mecânicas, molhabilidade e intumescimento. Por fim, avaliou-se a permeabilidade do fluconazol imobilizado em diferentes matrizes poliméricas através da mucosa vaginal utilizando o modelo da câmara de Franz e realizaram-se ensaios de atividade *in vitro* para a comprovação dos benefícios de se associar timol com fluconazol.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Desenvolver filmes poliméricos vaginais contendo fluconazol e timol para o tratamento da candidíase vulvovaginal resistente.

2.2 OBJETIVOS ESPECÍFICOS

- Desenvolver filmes poliméricos vaginais de quitosana através da técnica clássica de evaporação do solvente;
- Caracterizar os filmes poliméricos através de análises térmicas (calorimetria exploratória diferencial e termogravimetria), espectroscópicas (FT-IR), cristalográficas (DRX), morfológicas e quanto às propriedades mecânicas;
- Avaliar as características de superfície dos filmes poliméricos através de medidas do ângulo de contato em um goniômetro;
- Avaliar, gravimetricamente, como diferentes composições poliméricas afetam a capacidade de intumescimento dos filmes;
- Avaliar o impacto de diferentes matrizes biopoliméricas na permeabilidade vaginal do fluconazol bem como a retenção no tecido utilizando o modelo de difusão da câmara de Franz;
- Avaliar como a composição dos filmes afeta a desordem de domínios proteicos e lipídicos do epitélio vaginal suíno através da espectroscopia na região do infravermelho;
- Avaliar a atividade antifúngica *in vitro* do fluconazol após imobilização em filmes poliméricos através do ensaio de difusão em placa e seu efeito antifúngico com o timol em cepas resistentes de *Candida*.

3 CAPÍTULO I – REVISÃO BIBLIOGRÁFICA

3.1 CANDIDÍASE VULVOVAGINAL

A candidíase é uma infecção fúngica causada pelo gênero *Candida*, principalmente pela espécie *C. albicans*, mas também pode ser causada por espécies não-albicans tais como *C. tropicalis*, *C. glabrata* e *C. parapsilosis* (PFALLER & DIEKEMA, 2007). Estes patógenos integram a microbiota natural da mucosa vaginal, porém, quando o hospedeiro tem um desequilíbrio em seu sistema imune, ocorre a multiplicação exacerbada dos fungos e invasão dos tecidos (JOHAL *et al.*, 2016). Indivíduos imunocomprometidos, com neutropenia, diabetes, submetidos a hemodiálise ou transplante de órgãos possuem maior risco de apresentarem este desequilíbrio. O uso indiscriminado de antibióticos e corticoides também pode contribuir para que este quadro infeccioso possa ser observado (PAPPAS, 2006).

A característica dimórfica (capacidade de apresentar-se na forma de leveduras esféricas ou hifas) das espécies de *Candida* é responsável pela sua capacidade de infectar células e causar a patogênese. As leveduras possuem maior interação do fungo com a superfície da mucosa e as hifas contribuem para a invasão da levedura para dentro do tecido (BURIK & MAGEE, 2001).

Dentre as infecções vaginais, a candidíase é a segunda infecção mais comum (CDC, 2023). Aproximadamente 75% das mulheres apresentam pelo menos um episódio de candidíase vulvovaginal em sua vida, 40-45% apresentam dois ou mais episódios e 10-20% apresentam quatro ou mais episódios anuais (a chamada candidíase vaginal recorrente). Anualmente, estima-se uma incidência global de 138 milhões de novos casos de candidíase vulvovaginal recorrente, com maior prevalência na faixa etária entre 24 e 35 anos (DENNING *et al.*, 2018).

3.1.1 Sinais e sintomas

As candidíases representam infecções oportunistas e endógenas, acometem principalmente as mucosas orofaríngeas e vaginais, mas podem ter um alcance em nível sistêmico. Os processos patológicos são representados por inflamação, formação de pus e resposta granulomatosa (PAPPAS *et al.*, 2006).

O quadro clínico da candidíase vulvovaginal inclui diversos sintomas como prurido, disúria, dor na relação sexual, edema vulvar, fissuras e maceração da vulva,

corrimento branco e grumoso e placas brancas recobrando a vagina e o colo uterino (BRASIL, 2022). Graus de ansiedade, depressão, autoestima e qualidade de vida são alguns dos principais gatilhos que contribuem para o aparecimento destes sintomas (KARASZ & ANDERSON, 2003).

Visto que os sintomas clínicos não são específicos, o diagnóstico laboratorial se torna essencial para identificar a causa da infecção, o patógeno e espécie envolvidos para que se possa definir o tratamento mais apropriado.

3.1.2 Tratamento

A candidíase vulvovaginal é tratada com fármacos antifúngicos das classes dos azóis, que incluem os imidazóis (miconazol, clotrimazol e cetoconazol) e triazóis (fluconazol, itraconazol e voriconazol). Estes fármacos atuam inibindo a síntese do ergosterol, um componente essencial da parede fúngica. A classe dos polienos, representada pela anfotericina B e nistatina, também é considerada para fins terapêuticos. Estes fármacos agem alterando a permeabilidade da parede fúngica (COSTA *et al.*, 2003).

A via oral ainda é a mais utilizada na prática clínica, porém, efeitos colaterais são comuns. Para o fluconazol, por exemplo, há relatos de hepatotoxicidade, dores de cabeça e interações medicamentosas com medicamentos tais como hipoglicêmicos orais, anticoagulantes cumarínicos, ciclosporina, terfenadina, teofilina, fenitoína, rifampicina e astemizol (GILHOTRA *et al.*, 2014). Ainda, a terapia oral é contraindicada para gestantes, sendo o tratamento local a única escolha (BRASIL, 2022).

O tratamento preconizado pelo Ministério da Saúde inclui duas abordagens principais. A primeira preconiza o tratamento local da infecção através de cremes e pomadas de miconazol ou nistatina. A segunda opção é uma alternativa a possíveis falhas na opção anterior e indica que o tratamento seja realizado por via oral com fluconazol ou itraconazol. Para os casos de candidíase vulvovaginal recorrente, há um terceiro esquema terapêutico, que inclui tratamento com fluconazol por via oral durante 6 meses (BRASIL, 2022). O Quadro 1 descreve com mais detalhes as opções de tratamento recomendadas pelo Ministério da Saúde.

Quadro 1. Tratamento da candidíase vaginal preconizado pelo Ministério da Saúde do Brasil.

Primeira opção	Segunda opção	Tratamento em gestantes	Casos recorrentes	Comentários
Creme com 2% de miconazol, via vaginal, 5 g por dia, por 7 dias OU Nistatina 100.000 UI, via vaginal, uma aplicação por dia, por 14 dias	Fluconazol 150 mg, via oral, dose única OU Itraconazol 100 mg, via oral, 2x ao dia, por 1 dia	Durante a gravidez, o tratamento deve ser realizado somente por via vaginal O tratamento oral é contraindicado na gestação e lactação	Mesmas opções do tratamento da candidíase vaginal, por 14 dias OU Fluconazol 150 mg, via oral, 1x ao dia, dias 1, 4 e 7, seguido de manutenção: fluconazol 150 mg, via oral, 1x por semana, por 6 meses	As parcerias sexuais não precisam ser tratadas, com exceção das sintomáticas. É comum durante a gestação, podendo apresentar recidivas devido a variações hormonais e/ou fisiológicas (pH) no período.

Fonte: Brasil (2022).

Na maioria dos casos, o tratamento das pacientes acometidas por candidíase vulvovaginal inicia-se sem diagnóstico laboratorial ou confirmação do agente etiológico causador da doença. Assim, um tratamento inadequado e inespecífico contribui para o aumento dos casos de resistência fúngica aos fármacos e falha terapêutica (SOARES *et al.*, 2018). Além disso, a resistência das espécies de *Candida* aos antifúngicos tem sido atribuída ao uso profilático, que inclui exposições prolongadas a baixas concentrações de antifúngicos (PONTÓN & QUINDÓS, 2006).

Neste contexto, a busca por novas alternativas terapêuticas e a substituição da rota oral pela local se faz necessária. Os óleos essenciais e seus componentes majoritários, como o timol, por exemplo, aparecem como alternativas a estes casos de resistência em uma série de estudos, os quais são frequentemente combinados com os azóis (JAFRI & AHMAD, 2020; PALMEIRA-DE-OLIVEIRA *et al.*, 2013). Além de alta atividade, são caracterizados por baixa toxicidade, o que os torna uma opção terapêutica atrativa (PALMEIRA-DE-OLIVEIRA *et al.*, 2015).

Formulações locais, por sua vez, trazem como vantagens a facilidade de aplicação, maior controle da liberação de fármacos, maior concentração local do

fármaco, aumento da eficácia, melhora da adesão ao tratamento e evita os efeitos sistêmicos indesejáveis (MACHADO *et al.*, 2015).

3.1.2.1 Mecanismos de resistência

A resistência de um microrganismo aos agentes antimicrobianos é apresentada como a persistência de uma infecção no hospedeiro mesmo quando o fármaco se encontra em uma máxima concentração no local de ação (PERFECT & COX, 1999). Os fungos possuem capacidade de formar complexos extracelulares conhecidos como biofilmes para escapar do sistema imune e dos fármacos antifúngicos.

O principal mecanismo de resistência aos azóis é resultante da superexpressão do gene ERG11, que codifica a enzima 14- α -desmetilase, a qual converte lanosterol em ergosterol, principal constituinte da parede celular dos fungos. Assim, o mecanismo de resistência leva à produção excessiva da enzima e, portanto, requer maior concentração do fármaco no interior da célula para que ocorra a ação (CATALÁN & MONTEJO, 2006). Ainda, observa-se a superexpressão dos genes CDR1 e CDR2 (*Candida drug resistance*), que codificam bombas de efluxo específicas para o fluconazol, cetoconazol e itraconazol (CHEW & THAN, 2016; ESPINEL-INGROFF, 2008).

A formação de biofilmes representa um grande desafio nos casos de resistência aos antifúngicos. São descritos como uma matriz polimérica, imersa em meio líquido, aderidos em uma superfície sólida constituída de células microbianas e substâncias poliméricas extracelulares (*extracellular polymeric substances* – EPS) (LANGER *et al.*, 2018). Após a adesão de células planctônicas no tecido do hospedeiro, há formação de microcolônias que produzem as EPS e expressam genes diferenciais, os quais contribuem para a conversão de leveduras em hifas. Como consequência, há a formação de uma estrutura tridimensional complexa que limita a ação do sistema imune do hospedeiro e de fármacos. Ainda, os biofilmes maduros conferem mudanças fenotípicas das células microbianas e expressão de genes de resistência (AL-FATTANI & DOUGLAS, 2004). Mesmo quando ocorre a ação de antifúngicos, uma fração de células latentes não é atingida e será responsável pela reinfecção do tecido (LANGER *et al.*, 2018).

3.2 ADMINISTRAÇÃO VAGINAL DE FÁRMACOS

Como já mencionado, a administração oral de antifúngicos no tratamento da candidíase vaginal apresenta limitações como efeitos adversos sistêmicos, interações medicamentosas e contraindicação a gestantes. Como o Ministério da Saúde do Brasil preconiza, o tratamento local é a primeira escolha. A mucosa vaginal tem recebido bastante atenção nos últimos anos tanto pela possibilidade de se obter uma ação local (foco desta dissertação) quanto sistêmica.

As formulações vaginais tópicas devem proporcionar um aumento da retenção do fármaco e distribuição no canal vaginal, completa solubilização do fármaco no fluido vaginal, interação com o patógeno e recuperação da mucosa e do ambiente vaginal ao término da ação (HUSSAIN & AHSAN, 2005). A escolha desta via de administração para antifúngicos traz diversas vantagens em relação a rota oral, como maior concentração local do fármaco, redução da dose administrada, maior controle da taxa de liberação do fármaco, aumento da eficácia antimicrobiana, evita o metabolismo hepático de primeira passagem e diminui ou até mesmo evita efeitos adversos.

3.2.1 Anatomia

A vagina é um órgão tubular, fibromuscular, com ligeira curva em formato de “S” que possui de 6 a 10 cm de comprimento (MACHADO *et al.*, 2015). A cavidade vaginal possui extensa área de superfície devido às pregas transversais (100-150 cm²), alta vascularização e baixa atividade enzimática (ARGENTA *et al.*, 2021).

O tecido vaginal é composto por diferentes camadas. A camada mais externa é o epitélio escamoso estratificado não queratinizado, que tem função de barreira frente a patógenos externos. É coberto por muco cervical e a sua espessura varia com a idade, níveis de estrogênio, ciclo menstrual e estado de saúde (ASHOK *et al.*, 2012; CARMELLA *et al.*, 2015). A lâmina própria encontra-se logo abaixo do epitélio, uma camada de tecido conectivo que possui fibras elásticas, células de defesa como neutrófilos e linfócitos, além de vasos sanguíneos e linfáticos (COSTIN *et al.*, 2011). As últimas camadas, camada muscular e túnica adventícia, conferem alta elasticidade para o canal vaginal (CUNHA-REIS *et al.*, 2016).

Apesar da inexistência de glândulas, o tecido vaginal é recoberto por muco cervical, um fluido que inclui secreções endometriais, cervicais e vestibulares, resíduos de urina e produtos de autólise celular, além de transudato da parede epitelial (CUNHA-

REIS *et al.*, 2016). É composto por 95% de água, mas também por carboidratos, sais orgânicos, proteínas, colesterol, lipídios, aminoácidos, enzimas e mucina. A mucina é a glicoproteína mais importante, pois possui um papel importante na estrutura e na mucoadesão desse fluido (BALOGLU *et al.*, 2009).

O pH da região vaginal varia de 3,5 a 4,5 e é essencial na prevenção de infecções por microrganismos patogênicos. Os *Lactobacillus* spp., bactérias da flora vaginal, transformam o glicogênio em ácido lático, mantendo o ambiente na faixa de pH acima mencionada (CHOUDHURY *et al.*, 2011).

3.2.2 Rotas de permeabilidade

Durante o desenvolvimento de sistemas para a administração vaginal, é essencial entender os mecanismos de transporte de fármacos pelo tecido vaginal a fim de garantir que alcancem o alvo. Os ativos devem primeiramente se dissolver no fluido vaginal para então se difundirem pelos tecidos.

A absorção dos fármacos pelo tecido vaginal pode ocorrer por três rotas distintas. O *transporte transcelular*, que ocorre através das células, é dependente do gradiente de concentração e as moléculas lipofílicas têm preferência por essa rota. O *transporte paracelular*, que ocorre através das junções entre as células, é priorizado por moléculas mais hidrofílicas. Ambos os transportes transcelular e paracelular ocorrem a favor de um gradiente de concentração e sem gasto energético. Por último, há o transporte vesicular ou mediado por receptor, que é utilizado para compostos ativos que apresentam maior massa molar, sistemas nanoparticulados, peptídeos, aminoácidos e açúcares (HUSSAIN & AHSAN, 2005).

3.3 FLUCONAZOL

3.3.1 Características físico-químicas

O fármaco fluconazol (FLU) é quimicamente denominado α -(2,4-difluorfenil)- α -(1H-1,2,4-triazol-1-ilmetil)-1H-1,2,4-triazol-1-etanol (Figura 1), com fórmula molecular $C_{13}H_{12}F_2N_6O$ e massa molar de 306,27 g mol⁻¹ (ANVISA, 2019).

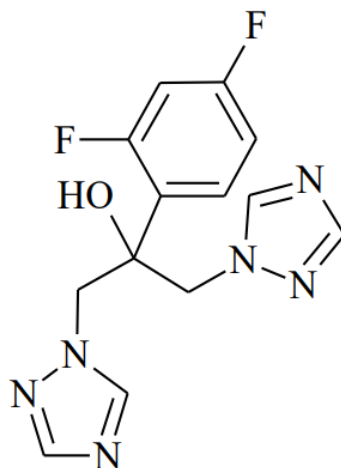


Figura 1. Estrutura química do fluconazol.

Apresenta-se como um pó branco ou ligeiramente branco, com faixa de fusão entre 138-140 °C. É solúvel em água (8 mg/mL a 37 °C) e facilmente solúvel em etanol e metanol (ANVISA, 2019; MOURA, 2008). Possui um log P de 0,5 e está incluído na classe I do Sistema de Classificação Biofarmacêutica, a qual engloba fármacos que apresentam alta solubilidade e alta permeabilidade (LINDENBERG *et al.*, 2004).

Formas polimórficas do FLU já foram relatadas na literatura. Gu & Jiang (1995), relataram as formas anidras, designadas I e II. Alkhamis e colaboradores (2002) relataram solvatos com acetona e benzeno e a forma monohidratada. Ainda, Caira *et al.* (2004) estudaram três polimorfos do FLU obtidos a partir da recristalização em 2-propanol, acetato de etila e água (CAIRA *et al.*, 2004).

3.3.2 Mecanismo de ação

O FLU é um fármaco antifúngico da classe dos triazólicos de primeira geração. Seu efeito fungistático se dá mediante inibição da enzima 14- α -desmetilase no complexo citocromo P450. Assim, ocorre inibição da conversão de lanosterol em ergosterol, com a depleção conseguinte de ergosterol, acumulação de precursores e perda da integridade da membrana fúngica. Também provoca alterações metabólicas no complexo P450 humano após ser administrado oralmente, aumentando assim as interações medicamentosas e efeitos adversos (FICA, 2004). Isto explica os efeitos hepatotóxicos frequentemente observados.

3.3.3 Farmacocinética

Propriedades farmacocinéticas similares são encontradas quando o FLU é administrado por via oral e intravenosa, o que pode ter relação com sua alta absorção oral. De fato, sua biodisponibilidade oral é superior a 80% em humanos. Sua absorção oral não é afetada pela presença de alimentos ou pH gástrico e seu pico de concentração plasmática é atingido entre 1 e 2 h, o que indica uma rápida absorção. Entre 11 e 12% da quantidade de fármaco absorvido encontra-se ligado a proteínas plasmáticas (ZONIOS & BENNETT, 2008).

O FLU é metabolizado no fígado pela enzima CYP2C9 e se difunde facilmente pelos líquidos corporais, incluindo o líquido cefalorraquidiano. Em humanos, tem um tempo de meia vida de aproximadamente 30 h e sua eliminação é majoritariamente renal. Entre 60 e 80% da dose administrada é excretada de forma inalterada na urina, o que pode ter relação com sua alta solubilidade (DEBRUYNE, 1997).

3.3.4 Efeitos adversos

Estudos epidemiológicos sugerem que a maioria das pacientes diagnosticadas com candidíase vulvovaginal por *C. albicans* resistente ao FLU tiveram uma exposição anterior a este fármaco (MARCHAIM *et al.*, 2012). Além dos mecanismos de resistência já mencionados, a falha terapêutica também se dá por abandono do tratamento devido aos efeitos adversos encontrados.

Ao ser administrado pela rota oral, pode causar hepatotoxicidade, dores de cabeça, anafilaxia, fototoxicidade, cardiomiopatia e distúrbios gastrointestinais (NSENGA & BONGOMIN, 2022).

Topicamente, o uso excessivo do FLU tem causado efeitos adversos como edema, irritabilidade e dor vulvar (PHILLIPS, 2005). Pacientes grávidas correm maior risco de desenvolver a candidíase vaginal devido a variações hormonais. Na gravidez, a administração oral do FLU foi associada a um aumento significativo no risco de aborto espontâneo em comparação a mulheres não expostas e mulheres com exposição tópica de azóis (MØLGAARD-NIELSEN *et al.*, 2016).

3.4 TIMOL

3.4.1 Características físico-químicas

O monoterpeno aromático timol (Figura 2) é o componente majoritário do óleo essencial de várias plantas pertencentes à família Lamiaceae (*Thymus*, *Ocimum*, *Origanum* e *Monardagenera*) e outras plantas como as pertencentes às famílias Verbenaceae, Scrophulariaceae, Ranunculaceae e Apiaceae (MARCHESE *et al.*, 2016). É quimicamente denominado 2-isopropil-5-metilfenol, com fórmula molecular $C_{10}H_{14}O$, massa molar de $150,22 \text{ g mol}^{-1}$ e log P de 3,43. Apresenta-se como um sólido cristalino a temperatura ambiente e possui ponto de fusão e ebulição de 51 e 233 °C, respectivamente (CHEN *et al.*, 2016; HAYNES, 2015). É solúvel em álcoois, soluções alcalinas e solventes orgânicos devido à desprotonação do fenol, mas é ligeiramente solúvel em água (0,9 mg/mL a 20 °C) em pH neutro (MEERAN *et al.*, 2017).

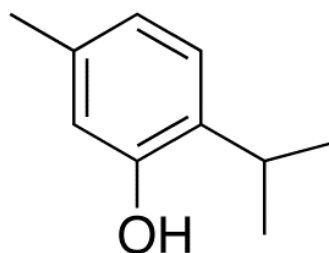


Figura 2. Estrutura química do timol.

3.4.2 Promoção da absorção

Considerando as propriedades de barreira mucosais e a necessidade de o fármaco alcançar o sítio de ação, promotores químicos de absorção podem ser alternativamente incluídos nas formulações. Estes agentes devem alterar temporariamente as propriedades de barreira do tecido, sem causar danos irreversíveis, aumentando a taxa de penetração do fármaco. Interações com domínios proteicos das células epiteliais, extração de lipídios intercelulares ou aumento da solubilidade do fármaco no veículo são alguns dos principais mecanismos envolvidos no aumento da permeação (MACEDO *et al.*, 2020).

O uso de terpenos como promotores de absorção já é bem documentado (CHEN *et al.*, 2016). Em um modelo de pele humana, por exemplo, o timol (1 – 4%) aumentou entre 3,1 e 17,2 vezes a permeação da corticosterona, o que foi atribuído a uma maior fluidização lipídica (CHANTASART *et al.*, 2009). Em outro estudo com pele de rato, a

presença de 5% de timol aumentou a permeação do meloxicam em 593 vezes (CHANG *et al.*, 2007). Em um modelo de mucosa bucal, a adição de timol (3%) em filmes de álcool polivinílico aumentou a absorção do fármaco benzonidazol em aproximadamente seis vezes (AMARAL *et al.*, 2021). Não foram encontrados estudos utilizando este terpeno como promotor de absorção em formulações vaginais, mas há relatos que a permeabilidade vaginal e bucal de fármacos é bem parecida, visto que histologicamente estas mucosas possuem estrutura similar (CHUCHUEN *et al.*, 2013). Outra vantagem é que terpenos apresentam baixa toxicidade, como já mencionado.

3.4.3 Atividades biológicas

Atividades antibacteriana, antifúngica, anti-inflamatória, antioxidante, larvicida, analgésica, anticonvulsante, anti-epileptogênica, cicatrizante, anti-hemolítica, atividade radioprotetora já têm sido descritas para o timol. Estas propriedades têm sido atribuídas ao farmacóforo fenol da sua estrutura. Compostos contendo os grupos fenólicos têm sido reconhecidos por conferirem proteção frente aos efeitos deletérios dos radicais livres tanto pela absorção ou neutralização dos radicais livres quanto pelo aumento de antioxidantes endógenos (MEERAN *et al.*, 2017).

Karpiński (2020) descreve que o mecanismo de ação antimicrobiana do timol é multidirecional, causando desde perturbações de membrana até inibição da síntese proteica ou do material genético, o que o torna um candidato ideal para uso combinado com outros antimicrobianos. Compostos lipofílicos acessam a parede celular com maior facilidade, causando danos a polissacarídeos, ácidos graxos e fosfolipídios, resultando no aumento da permeabilidade da membrana microbiana. Isto causa um desequilíbrio nos cátions H^+ e K^+ , afetando o pH e, subsequentemente, o funcionamento das organelas. Ainda, podem inibir a síntese de DNA, RNA, proteínas e polissacarídeos dos fungos, bem como desintegrar a membrana mitocondrial (KARPIŃSKI, 2020).

Nos últimos anos, estudos têm mostrado uma atividade anti-*Candida* para o timol. Jafri & Ahmad (2020) demonstraram que o óleo essencial da espécie *Thymus vulgaris* (0,39 $\mu\text{g/mL}$) apresentou sinergismo de ação com o fluconazol frente a *C. tropicalis*. O timol (0,19 $\mu\text{g/mL}$), por sua vez, apresentou uma ação sinérgica frente a *C. albicans*. Ainda, observou-se a inibição *in vitro* da formação de biofilmes, impedindo a adesão ao tecido (JAFRI & AHMAD, 2020).

A espécie *Thymus capitatus* (*Thymbra capitata*), que é caracterizada por alta concentração de timol e carvacrol em seu óleo essencial, também mostrou ser ativa tanto frente a células planctônicas quanto biofilmes de *Candida*. Um hidrogel de quitosana contendo óleo essencial de *T. capitatus* (0,17%) foi desenvolvido para o tratamento local de candidíase vaginal e, quando comparadas as concentrações inibitórias mínimas (CIM) do óleo e da quitosana isolados e em combinação, a CIM do óleo foi reduzida pela metade em testes com *C. albicans* e *C. glabrata* (PALMEIRA-DE-OLIVEIRA *et al.*, 2013).

3.5 FILMES POLIMÉRICOS

Considerando as limitações envolvendo formulações semissólidas e as vantagens que poderiam ser alcançadas com sistemas sólidos, o objetivo primário desta dissertação foi desenvolver filmes poliméricos contendo uma combinação de fluconazol e timol a fim de tratar cepas resistentes de *Candida* spp. Os filmes poliméricos têm sido amplamente utilizados na administração vaginal de fármacos já que evitam o metabolismo de primeira passagem e são capazes de controlar eficientemente a liberação de fármacos (CALVO *et al.*, 2019; DOLCI *et al.*, 2020; NEMATPOUR *et al.*, 2020). São membranas finas, hidrofílicas ou hidrofóbicas, compostas de uma ou mais camadas poliméricas, em que os ativos estão dissolvidos ou dispersos, e a partir da adesão à mucosa, proporcionam uma liberação local ou sistêmica dos fármacos (NOTARIO-PÉREZ *et al.*, 2020). Além dos polímeros, a composição dos filmes pode incluir plastificantes, desintegrantes, solubilizantes e estabilizantes (NEVES & SARMENTO, 2017).

O perfil de liberação de ativos a partir da matriz polimérica pode ser modificado para atender as necessidades de cada tratamento. Ensaio de liberação são úteis para guiar e prever alterações necessárias na composição destas formulações. Idealmente, os filmes devem ser flexíveis, não-tóxicos, biocompatíveis e resistentes aos movimentos do fluido vaginal (MANSURI *et al.*, 2016).

O uso de polímeros mucoadesivos é essencial para aumentar o tempo de residência das formulações na mucosa. A mucoadesão envolve um estágio de contato e a consolidação. O primeiro estágio envolve fenômenos de molhagem e intumescimento enquanto o segundo envolve a interpenetração do polímero na mucosa (MANSURI *et*

al., 2016). Assim, quanto menor e mais flexível for o polímero, mas será o grau de interação (adesão).

Os filmes poliméricos podem ser projetados para apresentar diferentes tamanhos e espessuras, a produção é barata e escalonável, podem ser facilmente removidos quando necessário e ficam aderidos na região de aplicação por períodos maiores que formulações semissólidas tais como géis e pomadas. Por serem formas sólidas, possuem baixo teor de água, e conseqüentemente, baixo risco de contaminação microbiana, além de evitar liberação do fármaco durante o armazenamento como ocorre com os sistemas nanoparticulados (JALIL *et al.*, 2019; ROSENBERG & DEVLIN, 2012).

3.5.1 Métodos de preparo

Diversos métodos têm sido empregados para preparar filmes poliméricos, como as técnicas de *eletrospray* e eletrofiação. Nesta técnica, há a aplicação de um campo elétrico em um capilar por onde flui uma solução de polímero de viscosidade controlada. À medida que a corrente elétrica acelera, o solvente evapora e as moléculas do polímero se movem na direção de um coletor, com a formação as fibras que compõem o filme. Ainda que escalonáveis, estas técnicas exigem um rigoroso controle de parâmetros como diâmetro capilar, tensão, vazão e distância até o coletor a fim de garantir baixas variações lote a lote (FENG *et al.*, 2019).

Filmes vaginais também podem ser obtidos pelo método de extrusão à quente. Neste método, polímeros são fundidos, adiciona-se o fármaco e outros componentes, os quais são misturados e forçados através de um orifício. Como este processo de extrusão emprega altas temperaturas (120–240 °C), apenas compostos ativos e excipientes resistentes ao calor podem ser utilizados, uma limitação deste método. Filmes uniformes e reprodutíveis são geralmente obtidos comparativamente a outros métodos (REGEV *et al.*, 2019).

A técnica de evaporação de solvente é a mais utilizada em escala laboratorial e por isto foi priorizada neste trabalho. Nesta técnica, o polímero e demais componentes são inicialmente solubilizados em um dado solvente, que pode ser água ou algum solvente orgânico (etanol é o mais utilizado por razões de toxicidade). Após a formação de uma solução homogênea, o conteúdo é vertido em um molde e o solvente é evaporado a uma temperatura pré-definida ou em temperatura ambiente com ou sem controle da umidade do ambiente. Isto faz com que as moléculas de polímero se

orientem e se entrelacem, formando um filme sólido (Figura 3) (NOTARIO-PÉREZ *et al.*, 2020). Sempre que possível, deve-se dispensar o uso de solventes orgânicos para evitar a presença de resíduos nos filmes. Dependendo do solvente escolhido, cristalização de fármacos pode ser observada, um fenômeno que também tem relação com a taxa de evaporação deste solvente (TIAN *et al.*, 2019).

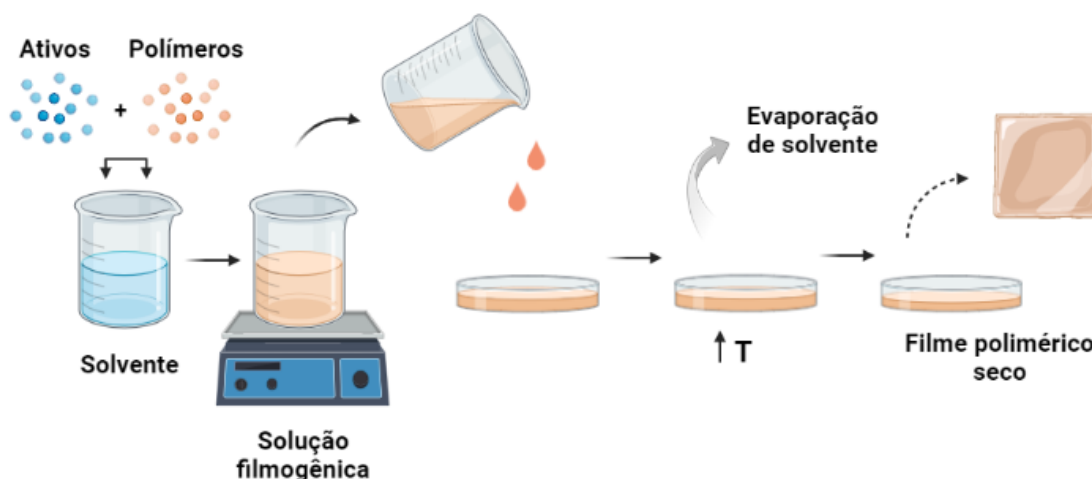


Figura 3. Técnica de evaporação de solvente. Criado a partir de Biorender (2022).

3.5.2 Polímeros

3.5.2.1 Quitosana

A quitosana é um polissacarídeo derivado do processo de desacetilação parcial da quitina, um biopolímero encontrado no exoesqueleto de crustáceos e moluscos. A estrutura da quitosana é formada por unidades de *N*-acetil-glicosamina e *N*-glicosamina (Figura 4) (SANAP *et al.*, 2022). Devido a presença dos grupos amino na sua estrutura, o pH do meio pode alterar o estado de ionização da quitosana, o que a torna um polímero catiônico natural (ÁVILA *et al.*, 2012). É uma base fraca ($pK_a = 6,51$) e, portanto, estes grupamentos são protonados em condições ácidas, tornando-a solúvel. Em altos valores de pH, torna-se insolúvel.

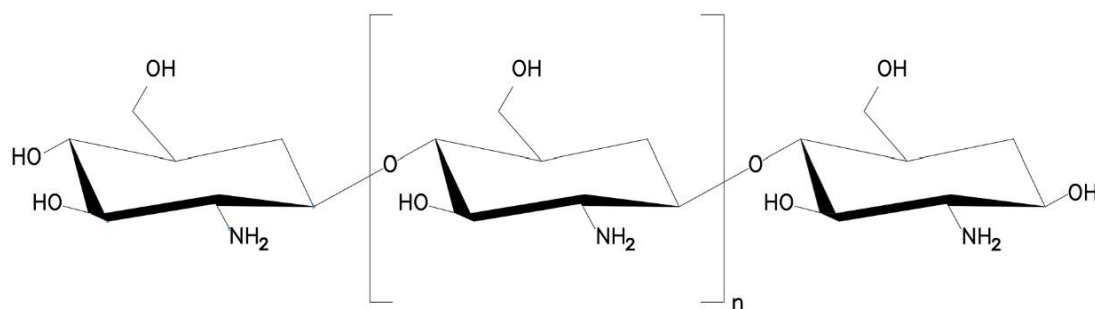


Figura 4. Estrutura química da quitosana desacetilada.

Por ser biodegradável, biocompatível e apresentar propriedades formadoras de gel e filme, a quitosana tem sido amplamente estudada no campo farmacêutico (ÁVILA *et al.*, 2012; CALVO *et al.*, 2019; SANAP *et al.*, 2022). Além de seu uso na modulação da liberação de fármacos, atividade antifúngica e antibacteriana tem sido atribuída a este biopolímero (ELIEH-ALI-KOMI *et al.*, 2016). Grau de desacetilação, peso molecular, pH do meio e temperatura são alguns aspectos que afetam suas propriedades. O mecanismo de ação antimicrobiana não é bem conhecido, mas sugere-se uma alteração da permeabilidade celular dos microrganismos mediante interação eletrostática (CHIEN *et al.*, 1998).

A aplicação da quitosana em filmes vaginais se torna interessante visto que os grupamentos amino e as hidroxilas da sua estrutura formam ligações fortes com a mucina, a glicoproteína que recobre o muco vaginal. Assim, a quitosana proporciona um efeito mucoadesivo essencial para este tipo de aplicação (PELEGRINO & SEABRA, 2017). Apesar de a quitosana possibilitar a obtenção de membranas e filmes adequados, sua resistência mecânica é muito baixa. Uma alternativa a esta limitação é a combinação de quitosana com outros materiais, como plastificantes e outros polímeros (ÁVILA *et al.*, 2012; RICCIO *et al.*, 2022).

3.5.2.2 Pectina

A pectina é um polímero natural ramificado extraído de diferentes partes de plantas e frutas tais como o bagaço da maçã e cascas de frutas cítricas. A funcionalidade da pectina nas plantas se dá pela contribuição à firmeza e integridade da estrutura do tecido vegetal (ESPITIA *et al.*, 2014). Devido a estas propriedades e alta solubilidade em água, a pectina tem sido muito utilizada no preparo de filmes poliméricos destinados

a aplicações farmacêuticas. Além da ampla disponibilidade na natureza, é biodegradável e biocompatível (RAMPINO *et al.*, 2016).

A pectina é um polissacarídeo aniônico constituído de polímeros lineares de ácido α -(1-4)-D-galacturônico (Figura 5) com açúcares neutros como L-ramnose inseridos ou anexados às cadeias principais (SOPYAN *et al.*, 2022). Segundo Mata e colaboradores (2009), o pKa da pectina pode variar de 3,6 a 4,5. As propriedades deste biopolímero tais como solubilidade e gelatinização são afetados pelo grau de metoxilação (GM) (LAZARIDOU & BILIADERIS, 2020). O GM é uma proporção de grupos carboxílicos que estão na forma esterificada. Pectinas que possuem mais de 50% de grupos carboxílicos esterificados são classificadas como alto GM e com valores menores que 50% como baixo GM (ESPITIA *et al.*, 2014).

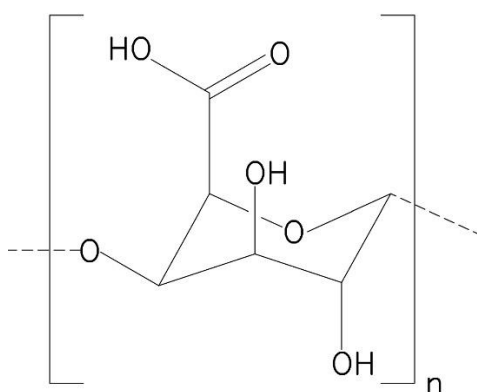


Figura 5. Estrutura química do ácido poligalacturônico, unidade formadora da pectina.

Os filmes de pectina são frágeis, o que dificulta a aplicação na mucosa vaginal. A fim de melhorar as suas propriedades mecânicas, a pectina tem sido combinada com outros polímeros tais como a quitosana (NESIC *et al.*, 2022). A pectina e a quitosana podem formar complexos polieletrólitos. Como a quitosana é uma base fraca e a pectina é um ácido fraco, em solução com pH específico, ocorre a interação eletrostática entre o grupo amino carregado positivamente (NH^{+3}) da quitosana e o grupo carboxila carregado negativamente (COO^-) da pectina (SOPYAN *et al.*, 2022).

3.5.2.3 Acetato succinato de hidroxipropilmetilcelulose

A celulose é o biopolímero mais abundante no mundo, o que tornou sua derivatização um processo barato e amplamente estudado. Os derivados de celulose

auxiliam na solubilização, liberação e evitam a cristalização dos fármacos (CHAVAN *et al.*, 2019). Permitem o desenvolvimento de filmes poliméricos com qualidade superior à de outros biopolímeros, além de apresentarem uma alta biocompatibilidade (KONDAVEETI *et al.*, 2017).

A hidroxipropilmetilcelulose (HPMC) tem destaque neste grupo como um polímero formador de filme por apresentar características de espessamento, inchaço e miscibilidade desejáveis para carreadores de fármacos. Dentre os polímeros modificados, o acetato succinato de hidroxipropilmetilcelulose (HPMCAS) é considerado um polímero de alta performance. Os grupos acetato e succinato são capazes de conferir caráter anfifílico (Figura 6), permitindo a incorporação tanto de fármacos hidrofílicos quanto hidrofóbicos. Interações eletrostáticas, hidrofóbicas e ligações de hidrogênio podem ocorrer entre fármaco e polímero. Os grupos ionizados do polímero aumentam a solubilidade em meio aquoso, enquanto a presença dos grupos hidrofóbicos resulta na formação de agregados coloidais (CHAVAN *et al.*, 2019; REHMAN *et al.*, 2019; WAISCZIK, 2020).

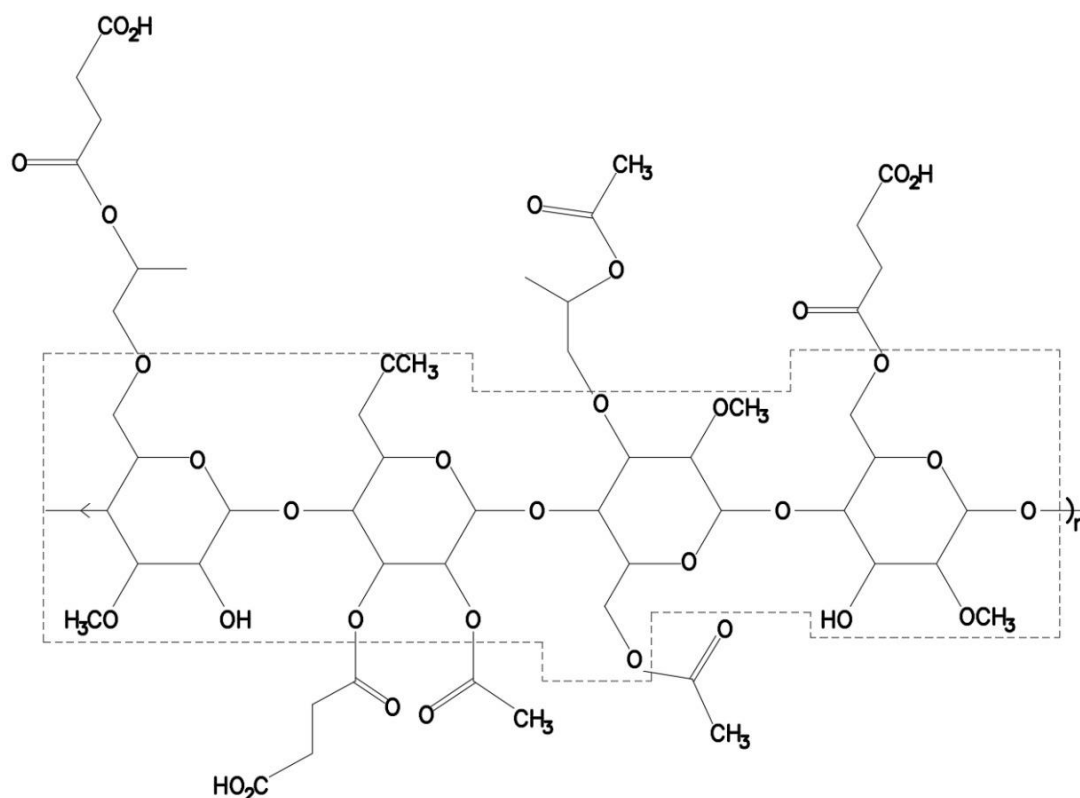


Figura 6. Estrutura química do acetato succinato de hidroxipropilmetilcelulose.

Isoladamente, o HPMCAS forma filmes rígidos e com baixa capacidade de intumescimento. As blendas poliméricas aumentam o volume livre e, como consequência, a flexibilidade das cadeias (WAISCZIK, 2020). Ainda, os grupos succinato possuem rotação livre, o que pode modificar as propriedades mecânicas dos filmes poliméricos, proporcionando maior flexibilidade e maleabilidade que a HPMC. Essa estratégia confere ao filme alta capacidade de adsorção de água, controle da liberação de ativos e maleabilidade, características fundamentais para a aplicação na mucosa vaginal (SANAP *et al.*, 2022).

3.5.3 Caracterização físico-química

Após a escolha dos componentes e da técnica de preparo que será empregada, os filmes poliméricos devem passar por uma série de ensaios de caracterização com o objetivo de avaliar a compatibilidade fármaco-excipientes e seu desempenho. Inicialmente, na inspeção visual, avalia-se a cor, transparência, textura de superfície, uniformidade e flexibilidade. Trata-se de uma análise preliminar e qualitativa, sem critérios bem definidos. Por outro lado, análises mecânicas, térmicas e espectroscópicas fornecem dados mais robustos e confiáveis (NOTARIO-PÉREZ *et al.*, 2020).

3.5.3.1 Força de tração e percentagem de alongamento

Para a aplicação vaginal, os filmes devem se adaptar aos movimentos da superfície da mucosa. Os valores de força de tração e de alongamento são medidos através de uma curva de tensão-deformação, a qual é gerada por ensaios mecânicos em equipamentos onde o filme é tracionado e deformado até o rompimento. Estas propriedades determinam a flexibilidade e resistência ao rompimento e dependem principalmente do tipo de polímero selecionado para a formulação (TIAN *et al.*, 2019).

3.5.3.2 Análise térmica

A análise térmica é muito útil na análise das transições e interações entre os componentes dos filmes. A determinação da estabilidade térmica é avaliada pela análise termogravimétrica (TGA), a qual expõe a amostra a altas temperaturas em uma atmosfera controlada e inerte, determinando-se a variação da massa em relação a condição inicial. Assim, é possível determinar a degradação térmica do filme e dos

componentes isolados, obtendo-se informações sobre a interação entre eles (SAADATKHAH *et al.*, 2020).

A calorimetria diferencial exploratória (DSC) é uma análise que permite diferenciar transições de primeira e segunda ordem e conhecer diferentes eventos térmicos dos filmes poliméricos e seus componentes isolados tais como desidratação, temperatura de transição vítrea, entalpia de fusão e cristalinidade dos fármacos. A técnica avalia o fluxo de calor da amostra em comparação a um material de referência após ser submetido a uma taxa de aquecimento ou resfriamento específica. A interação entre os componentes dos filmes ocorre mediante deslocamento dos eventos térmicos (NOTARIO-PÉREZ *et al.*, 2020).

3.5.3.3 Espectroscopia na região do infravermelho (FT-IR)

A técnica de espectroscopia na região do infravermelho com transformada de Fourier (FT-IR) possibilita analisar interações polímero-fármaco, polímero-polímero bem como de outros componentes presentes. As moléculas absorvem energia na região do infravermelho ($4000\text{--}400\text{ cm}^{-1}$) e os átomos sofrem vibração ou rotação. Cada tipo de material possui uma identidade espectral específica (NOTARIO-PÉREZ *et al.*, 2020).

3.5.3.4 Intumescimento e ângulo de contato

O teste de intumescimento está relacionado com as propriedades de desintegração, hidrofobicidade e capacidade de gelificação dos polímeros. Nesta análise, os filmes são imersos em um líquido aquoso (ex.: fluido vaginal simulado) em condições fisiológicas (37 °C e pH 4,2) e então pesados em diferentes intervalos. O índice de intumescimento é calculado pela razão entre a massa inicial e em diferentes intervalos (DOLCI *et al.*, 2020).

Outra análise que avalia a hidrofiliabilidade/hidrofobicidade da superfície dos filmes é o ângulo de contato, que é determinado em um equipamento conhecido como goniômetro. Quanto menor o ângulo de contato na interface do filme sólido e gota de água, maior é a interação entre eles, indicando maior hidrofiliabilidade da formulação (YOO *et al.*, 2006).

3.5.3.5 Morfologia

A microscopia eletrônica de varredura (MEV) avalia a morfologia e homogeneidade dos filmes poliméricos (empacotamento polimérico). Microestruturas e cristais de fármaco na superfície do material polimérico podem ser observados com esta técnica. Ainda, é possível confirmar a espessura aproximada da membrana (LINDERT & BREITKREUTZ, 2017).

3.5.3.6 Mucoadesão e tempo de residência *in vitro*

Para garantir que as formulações permaneçam aderidas na mucosa por um tempo compatível com a ação, testes de mucoadesão e avaliação do tempo de residência são necessários. A força de mucoadesão pode ser determinada usando um texturômetro ou tensiômetro de superfície, em que se monitora a força necessária para o descolamento do filme da mucosa. O tempo de residência, por sua vez, consiste em fixar a mucosa vaginal sob uma superfície angular, colocar o filme desenvolvido na superfície da mucosa e através de um *stress* mecânico ou fluxo constante de fluido vaginal simulado, afere-se o tempo de desprendimento do filme da mucosa. Ainda, pode-se analisar o tempo de residência na mucosa através de um sistema vaginal dinâmico que avalia o volume de fragmentos dos filmes que se desprendem sob um fluxo contínuo (LINDERT & BREITKREUTZ, 2017).

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4 CAPÍTULO II – ARTIGO PUBLICADO

Este capítulo foi organizado na forma de uma revisão bibliográfica que inclui estudos de desenvolvimento de formas farmacêuticas sólidas “inovadoras” para o tratamento local da candidíase vulvovaginal. Anéis vaginais, filmes, esponjas e nanofibras poliméricas foram as formulações abordadas. Sistemas tradicionais tais como comprimidos vaginais não integraram o escopo desta revisão. Além de discutir aspectos tecnológicos, vantagens e desvantagens de cada forma farmacêutica, foram apresentadas informações sobre a patogênese da candidíase vulvovaginal, mecanismos de resistência da *Candida*, opções terapêuticas atualmente disponíveis e o uso da mucosa vaginal como rota alternativa para a administração de fármacos. O produto deste capítulo é um artigo científico, que foi publicado na revista *Pharmaceutical Research* (FI = 4,914) em novembro de 2022.

4.1 ARTIGO

**ADVANCED SOLID FORMULATIONS FOR VULVOVAGINAL
CANDIDIASIS**

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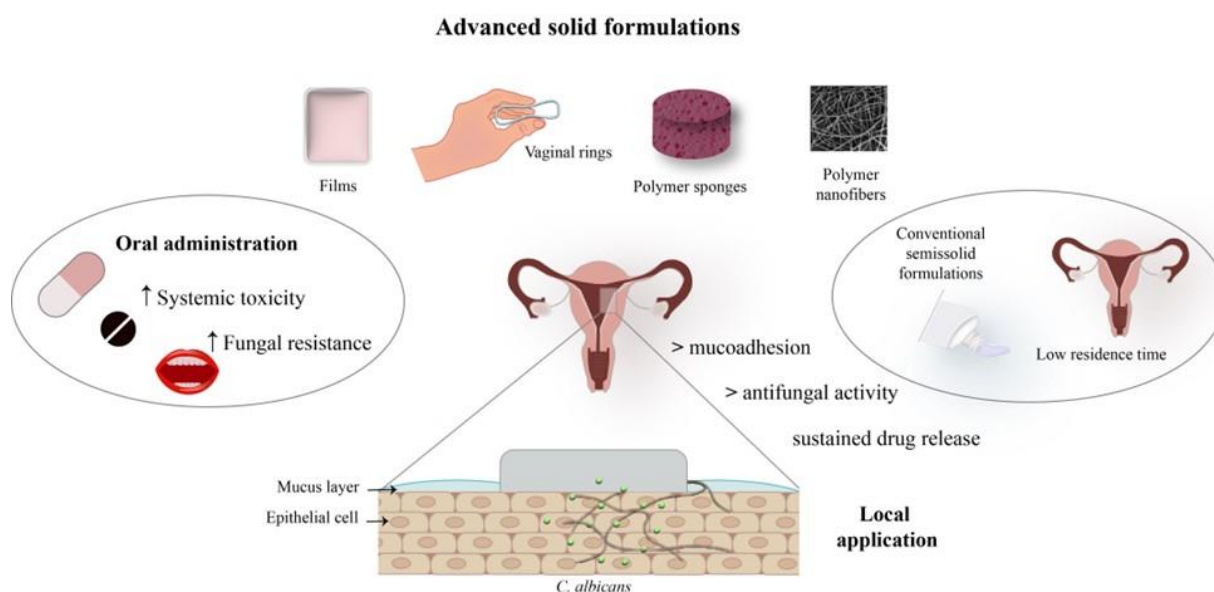
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ABSTRACT

Vulvovaginal candidiasis (VVC) is an opportunistic and endogenous infection caused by a fungus of the *Candida* genus, which can cause pruritus, dysuria, vulvar edema, fissures and maceration of the vulva. The treatment of vaginal candidiasis is carried out mainly by antifungal agents of azole and polyene classes; however, fungal resistance cases have been often observed. For this reason, new therapeutic agents such as essential oils, probiotics and antimicrobial peptides are being investigated, which can be combined with conventional drugs. Local administration of antimicrobials has also been considered to allow greater control of drug delivery and reduce or avoid undesirable systemic adverse effects. Conventional dosage forms such as creams and ointments result in reduced residence time in the mucosa and non-sustained and variable drug delivery. Therefore, advanced solid formulations such as intravaginal rings, vaginal films, sponges and nanofibers have been purposed. In these systems, polymers in different ratios are combined aiming to achieve a specific drug release profile and high mucoadhesion. Overall, a more porous matrix structure leads to a higher rate of drug release and mucoadhesion. The advantages, limitations and technological aspects of each dosage form are discussed in detail in this review.

Keywords: vulvovaginal candidiasis, solid formulation, vaginal formulations, drug delivery.

Graphical Abstract:



1. Introduction

Vulvovaginal candidiasis (VVC) represents one of the most common diseases in women seeking gynecological care, affecting approximately 75% of this population (1). It is caused by abnormal yeast growth in the mucosa of the female genital tract (2). The increase of opportunistic pathogens such as *Candida* is caused by environmental changes (3). In physiological conditions, *Lactobacillus* produces lactic acid, hydrogen peroxide and bacteriocins, maintaining the pH of the vaginal cavity between 4 and 5. This pH range avoids the overgrowth of pathogenic microbes. When this balance gets disturbed, a reduction of *Lactobacillus* spp. and overgrowth of *Candida* species is observed (4).

Vaginal irritation, vulvar burning, pruritus, erythema, dyspareunia and dysuria are typical symptoms of this disorder. A thin, white “cottage cheese-like” vaginal discharge with discrete pustulopapular peripheral lesions is associated with these symptoms (1). A more complicated form of VVC, known as recurrent VVC (RVVC), is defined when the patient has at least four confirmed episodes in a year with the clinical symptoms above mentioned (5). Every year, a global incidence of 138 million new RVVC cases is estimated, presenting the highest rates in the 24 to 35 age group (6).

VVC is mainly caused by the species *C. albicans*, but it can also be caused by non-albicans species such as *C. tropicalis*, *C. glabrata* and *C. parapsilosis* (7,8). *C. albicans* is considered the most pathogenic species for humans as it contains virulence

factors that include tissue adhesion, formation of hyphae and pseudohyphae, tissue invasion and biofilm formation (9). Biofilms are polymeric matrices adhered to a solid surface consisting of microbial cells and extracellular polymeric substances (EPS). In addition to changing the expression of resistance genes, these complex structures restrict the penetration of drugs and immune system cells within the biofilm architecture (10,11).

Another challenge in the therapy of this pathology is drug resistance. The prophylactic use, which includes prolonged exposures to low concentrations of antifungal agents, has been the main cause of this problem (12). Overexpression of genes that develop specific efflux pumps for some drugs such as azoles as well as overexpression of molecular targets also contributes to resistance to treatment (13). Once the treatment of patients usually starts without laboratory diagnosis and confirmation of the etiologic agent, therapeutic failures and cases of fungal resistance are common (14,15).

Vaginal candidiasis has been treated with azole drugs, including imidazoles (miconazole, clotrimazole and ketoconazole) and triazoles (fluconazole, itraconazole and voriconazole). Polyenes such as amphotericin B and nystatin have also been recommended (16). Therapeutic protocols include both systemic and local applications. Creams and ointments are the most traditional topical vehicles currently commercially available. The oral route is still the most used and has greater acceptance among patients; however, it is contraindicated for pregnant women and may result in several side effects and toxicity. Oral administration of fluconazole, for example, can lead to hepatotoxicity and headaches. Interactions with other drugs are also more frequent after an oral administration (17).

Given these disadvantages of oral administration and microbial resistance issues, the investigation of therapeutic alternatives and local treatments have been considered in many recent studies (18–20). The advantages of developing formulations with local action include ease of application, greater control of drug release and local drug concentration, increased efficacy, less incidence of systemic effects and thus greater probability of adherence to treatment (21). Topical vaginal formulations should provide increased drug retention and distribution in the vaginal canal, drug solubilization in vaginal fluids, effective interaction with the pathogen and recovery of the mucosa and the vaginal environment after treatment (22). In this context, advanced solid delivery systems such as vaginal rings, polymeric films, sponges and nanofibers

have been targeted in many studies. Solid formulations provide greater protection of the drug against degradation and reduce or avoid microbial contamination during storage as the water content is extremely low (23). Moreover, they avoid leakage of formulation from the vaginal cavity when compared to semi-solid formulations and can provide a more sustained and site-specific drug release (24). In this review, recent advances in solid formulations for VVC are discussed in detail. Various studies involving vaginal rings, polymeric films, sponges and nanofibers have been recently purposed, which may be justified by the high occurrence of the disease in women and the need to find solutions for microbial resistance. The advantages and disadvantages of each technology were also considered. Before discussing each formulation type, this review addressed pathogenesis of candidiasis, use of the vaginal mucosa as a route for drug delivery, and traditional local systems currently used in VVC treatment.

2. Pathogenesis of VVC

Candida albicans can colonize the human body without generating any sign of disease. As already mentioned, a physiological healthy microflora balance of *Lactobacillus* sp. and *Candida* sp. is found in the vagina, preserving the integrity of mucosal tissue barriers. The harmonic relationship of this microbiota protects the organism and avoids an infectious process triggered by opportunistic pathogens that colonizes the vaginal environment. When this balance gets disturbed, excessive growth of *Candida* species may be observed (4,25).

The infection rate is supported by diverse virulence factors, which included a morphological transition between yeast and hyphae forms, adhesin expression, secretion of hydrolytic enzymes and biofilm formation (3). The reversible morphological alternation between unicellular yeast cells and the filamentous phase (hyphae and pseudohyphae) represents an important virulence factor. Yeasts allow greater interaction of the fungus with the host and formation of hyphae, contributing to the invasion into the mucosa and preventing fungus cells are captured by macrophages and neutrophils (26). Filamentous forms of *Candida* species, in turn, are characterized by greater mechanical strength, increased resistance to phagocytosis, enhancing colonization and invasion of host tissues (8,27).

The transition from yeast to hyphae is affected by environmental conditions including temperature, CO₂ concentration and the presence of carbon sources (26).

Some hydrolytic enzymes play a crucial role in the adhesion, invasion, and destruction of host tissues such as phospholipase D, which is needed for yeast-to-hyphal transition in *C. albicans*. This enzyme hydrolyzes one or more ester bonds in glycerophospholipids, contributing to damage host cell membranes (10,27). Secreted aspartyl proteinase (SAP) is the enzyme most frequently implicated in *Candida* pathogenicity and the expression of some SAP genes (SAP4-6) particularly occurs during hyphal development (28).

The constituents of the cell wall of yeasts such as glucan, mannoprotein and chitin not only contribute to the structural shape of the cell, but they are also responsible for initiating a microorganism/environment interaction. Together with adhesins (fungal cell wall proteins), these agents represent the first pathogenicity mechanism due to interaction with specific ligands of the host cell (e.g., components of the extracellular matrix), leading to tissue invasion and infection (8,9).

The tissue invasion process by *Candida* spp. is followed by a series of reactions that finally damage the vaginal epithelium. Adhesion to the epithelial cells of the vaginal mucosa is the first step towards colonization and proliferation, involving several groups of proteins and genes such as genes associated with hyphae (Hwp 1), the integrin adhesion proteins (α m β 2, α v β 3 and α v β 5) and the ALS gene family (agglutinin-like sequence) encoding surface glycoproteins. The next step includes the penetration of hyphae to the vaginal epithelium through endocytosis or active penetration. Finally, the fungus passes through the vaginal epithelium, causing tissue damage by apoptosis or necrosis (4). Fig. 1 shows a schematic illustration of the tissue invasion process by two different mechanisms.

The formation of biofilms represents another relevant virulence factor. Yeasts can be attached to different types of host cells depending on the availability of carbohydrates, temperature, pH and production of extracellular enzymes. The adhesion and formation of biofilms initially occur by planktonic cells, which adhere to the host tissue randomly or by chemotaxis, or even by electrostatic and hydrophobic bonds with the surface (29). In the secondary phase, which is known as “irreversible adhesion”, yeasts form microcolonies able to produce EPS and express differential genes that change the structure architecture from yeast to hyphae. Finally, biofilm maturation occurs via multiplication and aggregation of new microorganisms, forming a three-dimensional structure (27,30). The complex and mature structures of biofilms restrict

the penetration of the host's immune system cells and drugs, resulting in phenotypic changes in microbial cells and the expression of resistance genes (11,31).

These virulence factors directly affect the vaginal tissue and can cause necrosis of the epithelium. Mucosal immune responses to *Candida* species play a key role in the management and control of infection after the hyphae invasion in vaginal epithelium, inducing pro-inflammatory innate immune responses, generating antimicrobial peptides, and activation of adaptive immunity. Thus, the vaginal mucosa becomes an intrinsic barrier to infection (28).

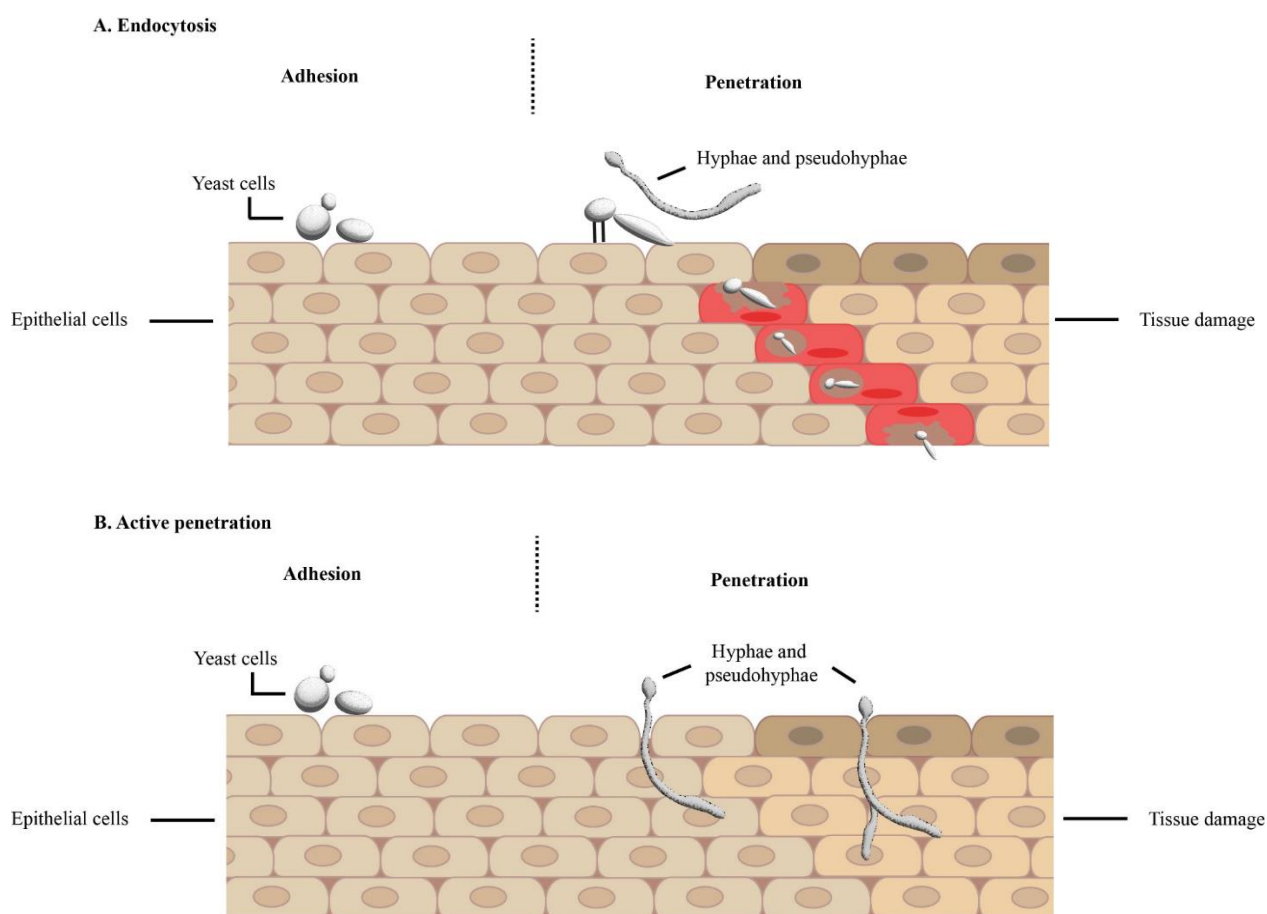


Fig. 1. Schematic representation of adhesion, invasion, and infection by *Candida* sp. by (a) endocytosis and (b) active penetration.

3. Composition of vaginal mucosa and drug transport mechanisms

The vagina is a longitudinally S-shaped, fibromuscular and collapsed tube in its relaxed state (21). Although the vagina is not composed of glands, its fluid is generated from mucus secretion of cervical columnar cells and transudation through

the vaginal wall. Secretions from the endometrium and fallopian tubes also contribute to the cervical mucus (22,32).

Mucus is a complex viscous adherent secretion composed of glycoproteins, inorganic and organic salts, mucins, proteins, cholesterol, lipids, carbohydrates, urea, enzymes, amino acids and high content of water (close to 95%), making it a highly hydrated system. Mucin is the most important glycoprotein of mucus, which is responsible for its structure and has been related to mucoadhesion (33). Considering that the mucus layer covers the vaginal mucosa, it affects the efficacy of formulations, resulting in product dilution and drug dissolution, increase leakage as well as reduction of drug residence time at the target tissue (32,34).

The vaginal wall is histologically composed of nonkeratinized stratified squamous epithelium, lamina propria, muscular layer and tunica adventitia (covering only their proximal segments) (35). The non-keratinized stratified squamous epithelium plays a crucial role in host defense and immune surveillance. It is the first cell layer that initially encounters environmental microorganisms such as the fungi of the *Candida* genus (36). The vaginal epithelial surface is coated by cervical mucus (32). Epithelial cells are at different stages of differentiation and are composed of 38 layers. The thickness of vaginal epithelium varies according to age, estrogen levels, menstrual cycle and health condition (37–39). In addition, the surface of the vagina is composed of numerous folds called as “wrinkles” or “rugae” providing support and an increased surface area for drug delivery (22,40).

Below the epithelium, a thin layer of connective tissue known as “lamina propria” is found. It is composed of numerous elastic fibers, polymorphonuclear leukocytes and lymphocytes. It is rich in blood and lymphatic vessels, which drain to the internal iliac vein (35,36). Lastly, the muscular layer provides high elasticity for the vagina due to the presence of smooth elastic fibers. The loose connective tissue of the tunica adventitia also helps to further increase elasticity (22).

The vaginal mucosa is characterized by a dense network of blood vessels and has been considered for both local and systemic approaches. In systemic delivery, drug needs to achieve the blood vessels (21). For a successful drug delivery to the target, drug dissolution in the vaginal fluid, epithelial penetration, and all factors associated with vaginal physiology should be considered.

The mechanisms of drug transport across the vaginal membrane can occur by diffusion through cells according to a concentration gradient (transcellular), diffusion

through tight a junction of cells (intercellular) and receptor-mediated transport (41). Transcellular and intercellular routes are both passive transport mechanisms. Lipophilic molecules are preferentially transported through the transcellular route whereas the hydrophilic counterpart follows a paracellular diffusion (42). Transmembrane proteins such as occludins, claudins and junctional adhesion molecules are found at the cell interface, restricting paracellular diffusion of molecules (43). In addition to the impact on drug transport, these routes are related to the invasion mechanisms of pathogens (44,45).

Physiological factors such as hormonal activity, pregnancy, microflora, age, enzymatic activity, cervicovaginal fluid pH, content and composition may affect the drug transport and distribution in the vaginal mucosa (46). An excessive fluid content, for example, provides a “washing” effect that decreases drug retention. Estrogens, in turn, stimulate epithelial cell proliferation and increase blood irrigation, transudation and cervical mucus production (47,48). Physicochemical properties of drugs such as pKa, log P, solubility and molar mass also may impact the drug transport (46). Low molar mass lipophilic drugs present a higher transport rate in vaginal mucosa than larger molecules or hydrophilic drugs (22). Changes in the composition of the vaginal epithelium affect the drug permeability in the mucosa as it depends mainly on the characteristics of the surface layer (e.g., thickness, cell tightness, lipid composition and organization) (44).

4. Therapeutic management of VVC

The main clinical goal of any VVC treatment is to treat the inflammation in the vaginal mucosa and eliminate the microorganisms responsible for the disease. Relief of signs and symptoms of the inflammation in vagina is usually achieved within 24-48 h whereas the mycological cure occurs 4-7 days after starting treatment (49).

The traditional treatment of VVC includes antifungals of the azole group such as fluconazole, voriconazole, itraconazole, ketoconazole, and miconazole, which are administered orally or topically. These agents act by inhibiting the synthesis of ergosterol, an essential component of the fungal wall (40,50). Polyene antifungals such as nystatin are usually used in topical treatment whereas azole drugs are in oral formulations. Nystatin has been considered less effective than azoles and reports of therapeutic failures are common. Polyene antifungals act by changing the permeability

of the fungal wall (16). The oral route is still the most used and has greater acceptance among patients. On the other hand, it is contraindicated for pregnant women and limitations in terms of several side effects and toxicity are common. Fluconazole is one of the most used drugs in VVC; however, resistance cases to *C. albicans* and non-*C. albicans* yeasts have been reported as the result of long-term therapies. Patients orally treated with drug preparations may also develop hepatotoxicity and headaches. Interactions with other drugs may reduce fluconazole efficacy (17,51,52).

The management of patients with complicated VVC and RVVC, mainly the patients with vaginitis caused by non-*C. albicans*, is difficult as the therapeutic options are poorly effective and side effects may be found (51). Therefore, more intense and prolonged therapeutic regimens are required in these situations, combining topical and oral therapies, multidrug regimens, or even the use of other active compounds (e.g., boric acid) (16,53). Essential oils appear in many studies as a therapeutic alternative to these complicated cases, which are often combined with azoles. Probiotics have shown a promising role in preventing VVC by recolonization of the vagina by *Lactobacillus* spp. (49). A new class of antifungals, the echinocandins, has been investigated due to the efficacy against azole-resistant strains and biofilm-forming strains. They act through non-competitive inhibition of the enzyme 1,3- β -glucan synthase, causing lysis of the cell wall (18). The investigation of new, effective, safe and low-cost antifungals has been also considered in various recent studies (20,50,54).

The high systemic toxicity of some antifungal agents and the local distribution of microorganisms causing VVC have also motivated the exploration of vaginal mucosa as a drug administration route. When compared to the oral route, vaginal mucosa avoids the first-pass effect in the liver and presystemic elimination within the gastrointestinal tract. Therefore, the drug dose used in formulation can be reduced. The vaginal mucosa also presents a dense network of blood vessels and a large surface area (34). Local administration is painless, enables self-insertion and removal of the dosage form, which increases patient adherence to treatment (33).

Conventional drug dosage forms such as creams, ointments, gels, tablets and suppositories are relatively effective, but they present several disadvantages compared to other advanced dosage forms. Leakage of formulation from the vaginal cavity in case of creams and gels, uncomfortable, low efficacy due to dosage inaccuracy, low retention in the vaginal epithelium, poor patient compliance, short interval between dosages,

prolonged duration of therapy, low bioavailability and uncontrolled drug release pattern are some of the main problems for these formulations (4).

Because of these limitations, recent studies in the pharmaceutical technology field have focused on development of drug delivery systems able to improve the distribution and retention of drugs in vaginal mucosa, provide greater patient's acceptability, a sustained drug release as well as increased activity and reduced toxicity. Bioadhesion is a key aspect of the successful application of these innovative systems in the vaginal cavity. Various polymers have been incorporated into solid systems to increase the contact time of the formulation on the vaginal mucosa.

5. Novel approaches in vaginal solid formulations

As already mentioned, the therapeutic arsenal currently available for the treatment of fungal infections is limited to polyene and azole antifungals and vaginal formulations that are not very effective in cases of microbial resistance are found on the market so far (51,55). Once the microorganism causing VVC is found on the surface of vaginal mucosa, topical formulations have been widely studied in the last years. Topical vaginal formulations provide increased drug retention and distribution in the vaginal cavity, high interaction with the pathogen and allow rapid recovery of the vaginal mucosa and environment after formulation removal (22,49).

Research and commercialization of vaginal solid formulations began in the 70s with a focus on hormonal and contraceptive activity (56). Since this moment, different vaginal rings have been marketed for this purpose (57). In recent years, vaginal rings, polymeric films, sponges and nanofibers have received attention with promising results in the local treatment of vaginal candidiasis. A summary of these solid formulations, their composition, manufacturing techniques and main results is presented in Table I. The study of different dosage forms allows that therapy can be customized according to the characteristics required by patients and active compounds (58). These systems may also reduce the dosing frequency and increase drug efficacy, which would reduce fungal resistance cases (59). The number of products based on new solid formulations has significantly increased, and this growth is expected to continue soon (40). The advantages and limitations of each advanced solid system were compared and presented in Table II.

5.1 Intravaginal rings

Intravaginal rings (IVRs) are toroid shape devices with approximately 5 cm in diameter composed of biocompatible polymer(s) and drug dispersion depends on ring type. IVRs offer advantages such as a controlled and extended release of drugs after a single application, user ability to control initiation and discontinuation, no interference in sexual activity and they are well-tolerated by the patients (60). Although IVRs have been firstly developed for hormonal therapy and contraception, a growing interest as a promising delivery system for microbicides against HIV, vaginal infections (VVC and bacterial vaginosis) and other sexually-transmitted diseases have been recently observed (61,62).

IVRs can be obtained from different polymer compositions and manufacturing processes. Various IVR designs are available according to the intended use, the duration of action and the number of drugs to be administered (Fig. 2). In the reservoir-type ring design, a drug-loaded core is surrounded by a rate-controlling polymer membrane (Fig. 2A). The sandwich vaginal ring design consists of a narrow drug-loaded polymer layer positioned between an inner central core and an outer membrane (Fig. 2B). In the matrix-type design, the drug is uniformly dispersed in the polymer matrix and drug release rates are proportional to both the drug loading and the surface area of the device (Fig. 2C) (61). Pod-type IVRs present several small drug-containing compartments known as “pods”, which can be loaded with the same or different drugs and present identical or different geometries (Fig. 2D). Each IVR design will result in a specific drug release profile (Fig. 3) (63).

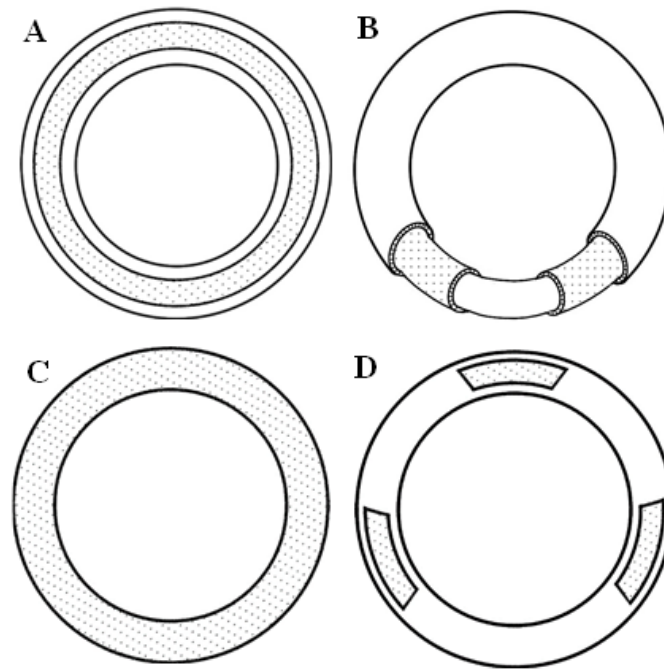


Fig. 2. Different intravaginal ring configurations: (a) reservoir-type; (b) sandwich; (c) matrix-type ring and (d) pod-type ring design.

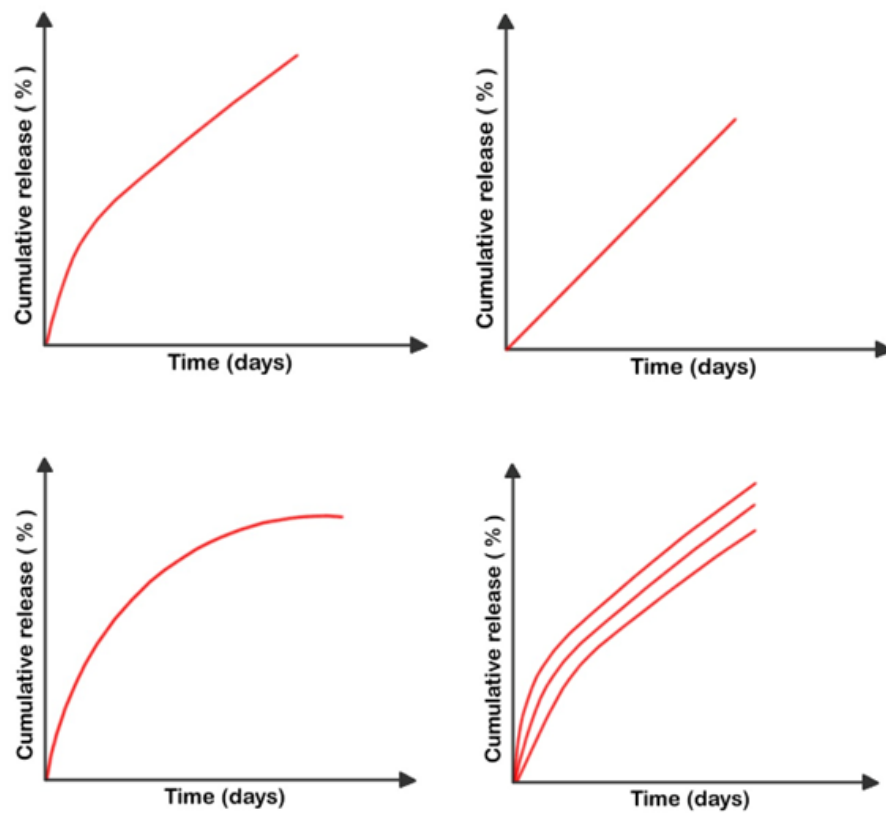


Fig. 3. Cumulative release profile of (a) reservoir-type ring; (b) sandwich; (c) matrix-type and (d) pod-type ring design.

Silicone devices or poly(dimethylsiloxane) were the first polymer materials used to prepare these systems. More recently, elastomeric polymers such as ethylene vinyl acetate and styrene have been prioritized as they result in more flexible vaginal rings (4). The selection of polymer type affects the final properties of the devices, drug solubilization and release profile. Thermoplastic polyurethanes (TPU), for example, can have different ratios of soft and hard segments, which result in copolymers with bi-phasic properties. These polymers offer a wide range of drug release properties useful in the development of IVR formulations for drugs presenting different physicochemical properties (64).

The development of IVRs for prophylaxis and topical treatment of VVC has gained attention in some recent investigations. Tiboni et al. (2021), for example, prepared clotrimazole-loaded IVRs by fused deposition modeling (FDM), which is one of the most extensively used layer-by-layer techniques for 3D manufacturing. After a computer-assisted design, thermoplastic materials are used to print 3D formulations presenting different shapes and dimensions, which can be prepared in a personalized way. In this study, clotrimazole (CTZ) was combined with TPU and castor oil to obtain a matrix-type device. The presence of oil was crucial to preparing extrudates in a single screw extruder, making the production step simpler. IVRs showed an elongation higher than 300% without breaking. After drug incorporation, the polymer degradation temperature was reduced from 316 to 297°C, suggesting a partial degradation of the TPU. *In vitro* release studies were performed in a 50% hydroethanolic solution and simulated vaginal fluid (SVF) containing 1% w/V of sodium lauryl sulfate at 37 °C. The daily drug release ranged from 0.94 to 5.05% in SVF and from 2.32 to 13.96% in 50% hydroethanolic solution, demonstrating a sustained drug release. After seven days, the cumulative release was 13.4% and 40.1% in SVF and 50% hydroethanolic solution, respectively. Although the amount of CTZ released is above the minimum inhibitory concentration for *C. albicans*, assays of *in vitro* activity were performed. In agar diffusion bioassays, IVRs loaded with 10% CTZ provided an area of growth inhibition of 2 cm. *In vitro* time-kill assays in simulated vaginal fluid showed complete growth inhibition of *C. albicans* after 5 days. The authors concluded that the developed IVR could improve patient compliance by decreasing the number of applications to a weekly application (65).

Silicone IVRs loaded with 10 pods of *Lactobacillus gasseri*, a commensal microorganism of human origin, were developed aiming to maintain the vaginal

microflora and avoid the overgrowth of *Candida* species. *In vitro* release assays were performed in phosphate-buffered saline solution for 21 days at 25 °C. The authors predicted that an amount between 0.6×10^4 and 20×10^8 of viable microorganisms should be released per day for this application. The developed system was considered suitable given that it was able to release between 1.1 and 14×10^7 microorganisms in this period. Changes in the size and number of pods could also be considered if adjustments in amounts of released viable cells are needed. Microscopic analyses showed the biofilm formation of *Lactobacillus gasseri*; demonstrating that microorganisms incorporated into vaginal rings could efficiently be converted into a viable form. Based on these results, the authors suggest that the developed device is useful in RVVC prophylaxis, but clinical studies are yet needed (66).

5.2 Vaginal films

Vaginal films are thin sheets, typically composed of one or several polymers, where the drug is found in a dissolved or dispersed form (58,67). The matrix-forming materials are mostly composed of polymers and plasticizers. These last agents are included to improve the mechanical properties of films, i.e., allow the film to adjust to the movements of the mucosal surface and simultaneously resist mechanical abrasion during application (68). Residual solvents and other agents acting as stabilizers, disintegrants or preservatives may be also found (69). A rapid disintegration and dissolution in body fluids may be achieved with this formulation type (67). Polymer films may be designed to present different sizes/dimensions, are easy and economical to manufacture, self-administered, avoid leakage and messiness in the vaginal cavity (compared to gels) and microbial contamination is reduced as the water content is minimal. When bioadhesive polymers are included in the formulation, high retention in the mucosa is observed (24,70,71). Unlike nanoparticulate delivery systems, polymer films prevent unspecific and premature drug release during storage (58,70).

The inherent properties of matrix-forming polymers (e.g., molecular weight) can affect the film's mechanical characteristics and drug release profile (72). Polymers should be non-toxic, non-irritant, devoid of leachable impurities, present appropriate wetting and spreadability, exhibit sufficient peel, shear and tensile strength. They can be classified according to their solubility (hydrophilic and non-water-soluble); structure

(one layer or multilayer, single-polymer or blends) or drug release mechanism (physical and biological stimuli-responsive films or matrix and reservoir-type films) (58).

Although the first vaginal films had been developed for contraceptive purposes (73), application in other vaginal disorders was rapidly observed in subsequent years due to their advantages (74). Vaginal films may be designed to provide a rapid or slow dissolution. A combination of polymers presenting different physicochemical properties is very useful when a sustained drug release should be achieved (24,67,68). In antifungal resistance cases, vaginal films have shown to be promising as they can release the drug in a specific amount and time (75).

Nystatin-loaded vaginal films were developed by solvent casting method selecting a carboxymethyl derivative of fenugreek gum as polymer and glycerol as a plasticizer. A derivatization step of this natural polymer was carried out to introduce an anionic character to an otherwise neutral polymer and thus enhance the interaction with vaginal mucous. An increase in the polymer concentration enhanced the mechanical properties of the film. The presence of glycerol (plasticizer), in turn, increased polymer-glycerol interactions and reduced polymer-polymer interactions, leading to a reduction and increase in tensile strength (TS) and elongation at break (EB), respectively. An increase in polymer and plasticizer concentrations also reduced contact angle values and resulted in an increased swelling index. The fast drug release found in a simulated dynamic vaginal system was explained by the high hydrophilicity of film formulations. All the incorporated drug was released within 5 h. *In vivo* antifungal activity studies revealed a reduction in the *C. albicans* counting from 10^5 to 3×10^2 colony-forming units (CFU)/mL within 14 days. Therefore, the developed system was considered promising as non-infected or asymptomatic microbial loads were found. Another positive aspect is that these systems were not irritant and toxic to vaginal mucosa (72).

This same research team developed a new polymer film with a similar experimental design by changing the bioadhesive polymer and plasticizer type. Tamarind seed polysaccharide (1-2% w/v) films were prepared by the solvent casting method and propylene glycol was selected as the plasticizer. Polymer concentration positively impacted on TS, % EB, water vapor transmission rate, swelling index and bioadhesion (increases the number of available groups for interaction). On the other hand, lower contact angle values were found with an increase in polymer concentration. Propylene glycol, in turn, increased the film hydrophobicity. After a screening to define the polymer and plasticizer concentrations, Tween[®] 60 was included as the solubilizer

agent and more assays were performed. This agent was crucial to reducing the drug crystallinity. The optimal film released 73.92% of nystatin at the end of 8 h in simulated vaginal fluid. The residence time of the formulation was found to be approximately 8.5 h. Although the authors had initially hypothesized that tamarind seed polysaccharide could play a synergistic effect with nystatin against *C. albicans*, this fact was not confirmed. Optimal polymer films and nystatin solution (control) showed a zone of inhibition of 1.1 and 1.5 cm, respectively. Formulations also maintained mass uniformity, thickness, folding endurance, drug content and % drug release after 6 months of storage (76).

The selection of chitosan has also been purposed to improve the residence time of formulations in mucosa regions. Calvo et al. (2019) developed tioconazole (TCZ)-loaded chitosan (CH) films with and without hydroxypropyl methylcellulose (HPMC) for VVC, considering polyethylene glycol 400 as the plasticizer. CH-based films with PEG 400 presented a higher elongation value than CH/HPMC films. On the other hand, films based only on CH showed greater swelling than CH-HPMC composite films, which was associated with the pH of simulated vaginal fluid (pH 4.2). All polymer films were able to swell for 24 h without suffering disintegration. Similar mucoadhesive strength was found among the tested formulations, which was attributed to overhydration provided by the polymers. Thermal and X-ray diffraction (XRD) analyses suggested that TCZ is found in an amorphous state inside the polymer matrix. In SEM analyses, CH-HPMC composite films showed a denser and more compact structure compared to CH films. All films showed a fast release of TCZ in distilled water (pH 4.3), reaching almost 100% of released drug within 60 min. Films loaded with TCZ showed greater activity against *C. albicans* than both the ovule and TCZ raw material, resulting in inhibition halos with diameters between 26 and 34 mm after 96 h assay. The amorphous state of the drug would explain this finding. The halo diameter reduced by 89% from 2 to 96 h for the TCZ ovules while the films showed reductions between 24 and 41%. This result suggests that the films provided a more sustained release of TCZ. Formulations based only on CH showed a certain degree of cytotoxicity, and thus CH-HPMC composite films should be prioritized (19).

Another study with chitosan-based vaginal films was performed by Real et al. (2013) (77). CH films with or without Carbopol® 971NF (CB) were prepared by solvent casting method for econazole nitrate (ECN) delivery. The performance of polyethylene glycol 400 and sorbitol as plasticizing agents was compared. Sorbitol resulted in more

flexible/elastic films than PEG 400. FTIR and thermal analyses suggested high chemical interaction between the polymers (COO^- group of CB would interact with the NH_3^+ group of CH); however, no interaction between these agents and the drug. In SEM and AFM analysis, few crystals on the film surface were observed, which was also attributed to the drug. CB/CH composite films also absorbed a higher amount of water (pH = 7.2) than films prepared only with CH (CB makes the film more hydrophilic and increases matrix erosion). In release studies, an initial burst effect was identified followed by slow diffusion. After 6 h assay, only 30% of ECN was released. After the film hydration, gel formation of CH causes clogging of pores, reducing the drug diffusion. The diameter of the inhibition halo of the commercial cream was half that of film formulations for both tested yeasts (*C. krusei* and *C. parapsilosis*). This improved activity of developed system was attributed to chitosan presence. The drug content of films was stable after 1 year of storage at 25 or 40°C and 58% RH (77).

Gelatin-based vaginal films were also considered to incorporate ECN in its free form or after its incorporation in solid dispersions (SDs). For SD preparation, polyvinylpyrrolidone (PVP), Soluplus[®] (amorphous material) and Gelucire[®] 50/13 (semi-crystalline material) were tested; however, SDs with this last agent increased the solubility more significantly (9.2 times compared to the pure drug) in pH 4.5. This solubility enhancement was attributed to the formation of micellar dispersions as the drug was found in a crystalline state in SDs. XRD patterns suggested an acid-base interaction between the drug and the polymer, which would result in the transformation of ECN into crystalline econazole (EC) after incorporation into gelatin films. The presence of Gelucire[®] 50/13 improved the film's mechanical proprieties and reduced the swelling rate in aqueous media. Cross-linking of gelatin with genipin (2% w/w) was also considered for comparison purposes in the subsequent assays (gelatin films are much hydrophilic). Genipin crosslinking did not affect the tensile properties of the films in relation to films containing Gelucire[®] SDs. On the other hand, genipin cross-linked films presented a more controlled drug release up to 24 h than uncross-linked films (presented greater burst release) in phosphate buffer. Although all polymer films controlled the drug release up to 24 h, only the uncrossed-linked systems showed to be able to achieve high drug concentrations (around 40%) at the site of application within the first 5 h. Both Gelucire[®] and genipin were effective in improving the bioadhesive properties of gelatin films. Gelucire[®]-based SD films displayed greater activity against *C. albicans* than gelatin films alone and systems crosslinked with genipin (inhibition

halo diameter of 30 mm, 20 mm and 23 mm, respectively) and thus they were considered more promising for the treatment of VVC (78).

Films composed of hydroxypropyl cellulose (HPC) and sodium alginate were prepared by solvent casting method for the delivery of clotrimazole. Given that films prepared only with HPC and sodium alginate do not provide desirable mechanical properties, the plasticizers propylene glycol (PG) and PEG 400 were tested. PG was more efficient in improving this parameter and thus it was considered in subsequent assays. Isopropyl myristate (IPM) was selected as an absorption enhancer. A rapid film disintegration was found (≤ 20 min), which increased with the polymer concentration. This parameter also affected the tensile strength significantly. The optimized vaginal film showed a faster drug release rate than the marketed product (Candid-V6[®] tablet). The film formulation released 70 and 83% of CTZ after 1 and 6 h, respectively. The marketed product, in turn, released 14 and 41% of the drug in the same intervals. In the activity assays against *C. albicans*, the inhibition halo values of optimal films and Candid-V6[®] tablets were 24 and 23 mm, respectively. The films did not affect the growth of *Lactobacillus* from the vaginal canal, preserving the physiological microflora. Therefore, a selective action of the developed delivery system would be achieved (79).

More recently, this same research team prepared vaginal films by the solvent casting method changing the polymer matrix (a mixture of chitosan and pectin) and the incorporated drug (fluconazole-FLZ). Glycerol was selected as the plasticizer and dimethyl sulfoxide as the permeation enhancer. X-ray diffraction study and thermal analyses confirmed cross-linking between polymers. The optimal film provided a greater drug release control than the marketed FLZ gel (Flucos[®]) in simulated vaginal fluid at pH 4.2 at 37 °C. In the developed film, 55.6% of the drug was released within 6 h while the marketed formulation released 74.5% of FLZ in the same period. At 24 h, the amount of FLZ released from the polymer film and Flucos[®] gel was 79 and 77%, respectively. Polymer films did not affect the growth of *Lactobacillus*, suggesting that they preserve physiological vaginal microbiota. Developed film and Flucos[®] gel formulations showed a similar *in vitro* inhibitory activity against *C. albicans* (zone of inhibition of 12 mm vs. 18 mm, respectively). *In vivo* studies performed on rabbits showed no signs of irritation or inflammation after 3 days of treatment (80).

Kumar et al. (2013) have also developed vaginal films loaded with FLZ (81). Film formulations were developed by solvent casting method using HPMC (1, 2, 3 and 4% w/w) and polyethylene glycol 400 as the polymer and plasticizer, respectively.

FTIR analyses suggested no chemical interaction between the drug and excipients. Non-uniform drug distribution was observed in the formulations with high HPMC concentration. All HPMC films showed significant flexibility. The tensile strength and elongation at break increased with the concentration of HPMC. *In vitro* release studies performed in acetate buffer (pH 4.6) showed that the drug release reduces with increasing polymer concentration. After 24 h, films containing 1, 2, 3 and 4% of HPMC released 99.94, 93.87, 88.31 and 66.50%, respectively. Therefore, formulations prepared with higher polymer concentrations were more effective in controlling the drug release. Only the formulation containing 1% of polymer released the drug through an anomalous transport mechanism whereas other formulations (2, 3 e 4% polymer) released the FLZ by a Fickian-type diffusion mechanism.

5.3 Vaginal sponges

Polymer sponges are defined as dispersions of gas (usually air) in a solid matrix to provide a solid porous structure. When they are hydrated by vaginal fluids, sponges assume a hydrogel structure for longer periods, presenting increased residence time and higher drug absorption. They also provide a higher drug loading capacity compared to the thin film formulations due to their porous nature and greater surface area (82). These systems can be easily prepared by freeze-drying technique due to the typical microporous structure, high liquid absorptivity and high surface/volume ratio (83).

In the case of VVC, the use of mucoadhesive polymers for sponge composition prolongs the drug residence in the vaginal cavity due to the formation of physical and chemical bonds with the mucosa. Chitosan, gelatin, cellulosic derivatives such as the HPMC and Carbopol® 934 represent the most used polymers (39). The development of sponges that are retained in the vaginal cavity and allow a prolonged drug delivery has been exploited in the last years, but only a few studies were designed to prepare a vaginal sponge-like dressing loaded with antifungal drugs for VVC (84–86).

The adhesion to the vaginal mucosa and rehydration with a small amount of vaginal secretion/fluid turning into hydrogel with adequate viscosity represents the main criteria to be observed during the development of new systems. In addition, the polymer sponges should be sufficiently robust and flexible to provide patient comfort and convenience. The mechanical strength and functionality of these formulations have been improved by combining polymers with different properties (87).

Vaginal polymer sponges loaded with butoconazole nitrate (BN) were developed by lyophilization technique for VVC applications. The matrix-forming polymers were chitosan, gelatin, HPMC and Carbopol® 934. Calcium chloride and sodium carboxymethylcellulose were selected as crosslinking and viscosity modifier agents, respectively. Tween® 80 or a mixture of Labrasol® and Transcutol®, in turn, were included as solubilizer agents. Chitosan sponges showed the highest tensile force values. The combination of HPMC and CH also resulted in high tensile strength values, which was attributed to the high degree of entanglement of polymers. In presence of calcium chloride or for gelatin/CH combination, sponges with undesirable mechanical properties were obtained (brittle/much rigid). CH sponges also presented highest mucoadhesion and swelling index. The high swelling index of the CH sponge contributes to enhancing polymer chain relaxation and interpenetration into the vaginal mucosa, explaining the greater residence time. SEM and thermal analyses suggested the presence of drug crystals in the formulations. *In vitro* release in simulated vaginal fluid at 37 °C revealed a burst release of BN within the first 30 min for optimal systems followed by a gradual increase. All polymer sponges were completely biodegraded within 24 h. Calcium chloride resulted in a slower initial drug release, which may be related to the cross-linked structure of the sponge. In agar disc diffusion susceptibility testing, HPMC/CH was more active against *C. albicans* than CH/gelatin sponges. A MIC value of 4.45 µg mL⁻¹ was found, demonstrating that formulation preserved the antimicrobial activity of BN (0.12-8 µg mL⁻¹). Sponges maintained the structure, drug content and *in vitro* release profile of BN upon storage for 3 months (86).

Plant materials have also been incorporated into polymer sponges for VVC. Ge and Tang (2016) initially prepared liposomes of tea tree oil (TTO) by the thin-membrane hydration method with sonication and then these systems were added to chitosan sponges, which were prepared by the freeze-dried method. This essential oil is extracted from the Australian native plant *Melaleuca alternifolia* and its activity against *Candida* spp is already well-known, which is attributed to the major compounds terpinen-4-ol and 1,8-cineol (88,89). Once the TTO is unstable to light, temperature and oxygen, the authors purposed the preparation of liposomes before incorporation in polymer sponges. The sponge porosity, water absorptivity, water retention capability and water vapor transmission rate decreased for increasing proportions of CH. A similar effect was observed when the proportion of TTO-liposome (TTOL) in sponges was increased. On the other hand, the increase in glycerol concentration and freezing rate

had an opposite effect on these parameters. The incorporation of TTO-loaded liposomes in the CH sponges improved the microbicide effect against *C. albicans*, achieving a microbial clearance rate greater than 80% after 8 min of treatment. Fungal membrane damages were identified in TEM analyses. The killing log values (KLV) provided by pure CH sponges were between 1 and 2 after 12 h, which increased to 3 after treatment with TTOLs/CH composite sponges. These systems could adsorb and intercept microbial cells through the internal pore and surface charge. They also destroy microbial intercellular substances, disperse cell colonies and damage the integrity of cell membrane, leading to the death of microbial cells (85).

Rossi et al. (2012), in turn, have purposed the application of polymer sponges based on peptides to treat fungal resistance. Antimicrobial peptides (AMPs) are short amphipathic peptides (15 to 50 amino acid residues) that have been considered for VVC due to high specificity and activity as well as reduced side effects. Defensins, secretory leukocyte protease inhibitors, calprotectin, lysozyme and lactoferrin are some examples of the families of AMPs (90,91). Mucoadhesive sponge-like dressings based on chitosan ascorbate (CA) or sodium hyaluronate/lysine acetate (HAS) combination were developed. CH is well-known due to its mucoadhesive effect whereas HAS combination was used aiming to improve the penetration of these peptides in the vaginal mucosa. Fluorescein isothiocyanate dextran (FD4) was used as the model molecule. All sponges demonstrated mechanical resistance suitable for this application as deformation force values greater than 15 N were obtained; however, a reduction in shear stress was found after dressing hydration. An increase in sponge hydration time also reduced the mucoadhesion. Chitosan with higher molecular weight resulted in greater bioadhesion. On the other hand, HAS dressings provided low mucoadhesion. Besides mucoadhesive and penetration enhancement properties, vehicles for peptides should protect them from enzyme degradation. For this reason, the ability of the developed dressings to inhibit carboxypeptidase A was evaluated. The enzyme activity inhibition provided by polymer sponges was significantly lower than the control. HAS sponges reduced the enzyme activity in 40% whereas CA sponges completely inhibited the enzymatic activity. HAS sponges released 90% of FD4 in acetate buffer pH 4.5 after 6 h. CA sponges, in turn, provided a sustained drug release, achieving only 30% of release FD4 in this time interval. CA-based dressings also provided a higher penetration rate for FD4 in vaginal mucosa than HAS-based systems (84).

5.4 Vaginal nanofibers

Polymeric nanofibers (PNs) have been extensively studied as vaginal delivery systems due to the fine diameters (range from submicron values to several nanometers), large surface area per unit mass, high porosity, high gas permeability and small pore size of the fiber (92). PN can be prepared by electrospinning and sol-gel processes for both local and systemic drug delivery (93). The large surface area helps in transmucosal interaction and permeation. Nanofiber materials can further be tailored to specific applications by changing the composition and surface characteristics (94). Traditional PNs are characterized by a burst release of drugs, resulting in a rapid washout of this molecule from the mucosal surfaces. In addition, intrinsic properties of PNs such as surface topography and porosity also affect drug diffusion. Thus, both system composition and preparation techniques should be carefully selected to extend the drug release (46,95).

Electrospinning has been considered one of the most efficient techniques used for the development of nanomaterials since the 20th century. In this technique, a high electrical field is applied between a metallic needle, which contains the polymeric solution or melt, and a grounded collector. The liquid ejected from the needle tip leads to the formation of the so-called “Taylor cone”, followed by elongation, thinning, and deposition of sub-micron fibers on the collector (96). An illustration of the electrospinning process is presented in Fig. 4. This method helps to convert drugs from a crystalline state to an amorphous state because of its flash evaporation and sudden expansion of the polymer, improving the drug solubility (97,98). Moreover, electrospinning is easy to scale up and is characterized by a simple operation, representing an economically viable alternative to preparing continuous and reproducible nanofibers (93,96).

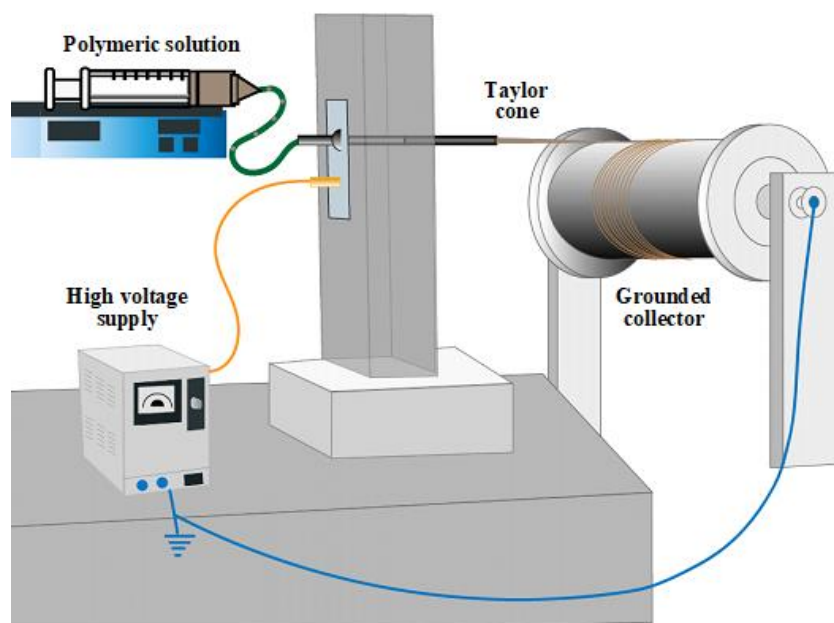


Fig. 4. Schematic representation of electrospinning process.

Amphotericin B-loaded PLGA nanofibers were developed by electrospinning for the local treatment of recurrent VVC. Amphotericin B has been recommended in cases of resistance to other antifungal drugs. In addition to biodegradability and biocompatibility properties, PLGA was selected because its degradation results in lactic and glycolic acids. These agents reduce the pH of the vaginal fluid, a positive effect to control the *C. albicans* proliferation. The drug was completely dispersed into the polymer matrix and induced the enlargement of the diameter of nanofibers. Each day, approximately 12.5% of amphotericin B was released in phosphate buffer solution pH 7.4, suggesting a sustained drug release. The drug release was completed at the end of the eighth day. Interestingly, no initial release burst was found, a common event for this formulation type. The *in vitro* antifungal activity of amphotericin B-loaded nanofibers against *C. albicans* and *C. non-albicans* (*C. krusei*, *C. glabrata*, and *C. tropicalis*) was tested through the microdilution test. Values of MIC and MFC (minimum fungicidal concentration) ranged from 0.25 to 1.0 mg/mL and 1.0 to 1.5 mg/mL, respectively. The growth inhibition provided by the nanofibers was higher than pure drug, which was associated with an extended drug release. In addition, a murine model of vulvovaginal candidiasis was considered for activity assays. After 72 h of a single application of the nanofiber formulation, a complete remission of the infection was found, which also was confirmed by histological analyses. No systemic toxicity in kidney and heart were

observed (organs that are normally affected by this drug), suggesting a local distribution of drug (54).

Polyvinyl alcohol (PVA)-based nanofibers obtained by co-electrospinning were selected for fluconazole incorporation. The study proposal was to develop local action nanofibers for VVC as oral fluconazole presents toxic effects associated with long-term therapies. The diameter of the optimal fibers ranged from 150 to 180 nm. Thermal analyses showed a slight decrease in the melting point of the drug in the electrospun nanofibers, suggesting a certain drug amorphization. Thinner fibers showed higher swelling degree, presenting an increase up to 4 h. After this period, swelling index values reduced, which may be attributed to polymer matrix erosion. Drug-loaded nanofibers exhibited improved mechanical strength compared to blank nanofibers. *In vitro* release studies of the nanofiber mats performed in goat vaginal showed an initial burst release of 35.13% of the drug within first 2 h (drug from the large nanofiber surface), followed by a sustained release of 97.8% for up to 6 h (drug present in the matrix core). Finally, the anti-Candida activity of nanofibers was evaluated. After 72 h, an inhibition zone of 1.2 and 4.8 cm was found for the pure drug and nanofibers, respectively. The system did not inhibit the *Lactobacillus acidophilus* growth, suggesting that it does not affect the physiological microflora of the vaginal epithelium (99).

Tuçcu-Demiröz et al. (2021) developed PVP nanofibers loaded with free benzydamine (BZY) and BZY lyophilized nanoparticles (NPs) through the electrospinning technique. BZY is an anti-inflammatory and antiseptic drug that has been considered to treat nonspecific vaginitis and VVC. Hydroxypropyl methylcellulose gel formulations loaded with free drug and BZY lyophilized NPs were prepared for comparison proposals. BZY-loaded chitosan/tripolyphosphate (TPP) nanoparticles incorporated into both vehicles (nanofibers/gel formulations) were prepared by the ionic gelation method. Optimal NPs showed a particle size, zeta potential, PDI and encapsulation efficiency of 128 nm, 14.1 mV, 0.220 and 95.04%, respectively. The presence of chitosan/TPP NPs in HPMC gels increased the degree of crosslinking, enhancing the viscosity. In nanofibers, the addition of NPs reduced both tensile strength and elongation at break values. NPs would reduce interactions between PVP polymer chains. They also increased the nanofiber diameter, which may be related to the reduction in the conductivity of the polymer solutions. A contact angle equal to 0° was found for the electrospun nanofibers, which suggests high wettability property. Thermal

analyses suggest an amorphization of BZY from NPs after incorporation into nanofibers. The addition of BZY NPs into a gel and nanofiber formulations reduced mucoadhesion properties when compared to formulations with the free drug. Nanofibers had lower mucoadhesive properties than that gel formulations, but were sufficient for vaginal application. After 24 h, the amount of drug released from nanofibers with and without drug nanoparticles was 53.03 and 109.24%, respectively. On the other hand, gel formulations with and without nanoparticles released 15.9 and 97.6% of BZY, respectively. Therefore, the presence of NPs reduced the release rate of BZY, providing a controlled release. *Ex vivo* permeability results support the results from the *in vitro* release study. After 48 h, nanofibers provided a higher permeability flux than the gel formulation due to the porous structure and large area surface (7.661 vs. 5.578 $\mu\text{g}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$). Taken together, the results suggest that nanofibers containing NPs are more suitable for vaginal applications than NPs-loaded gel formulations (100).

Another comparative study was performed by Nematpour et al. (2020) with CTZ-loaded polymer nanofibers and films composed of dextran and alginate. When used alone, alginate results in rigid and fragile nanofibers. For this reason, it was combined with polyvinyl alcohol for nanofiber composition. Propylene glycol (PG) was also included in film formulations as a plasticizer agent. Hybrid nanofiber presented a diameter of 492 nm, and the thickness of the film was close to 240 μm . XRD and thermal analyses suggest that CTZ-loaded film formulations are more crystalline than CTZ-loaded nanofibers. During the film formation, solidification takes a longer time, which results in greater crystallinity. Suitable mechanical properties were found for both nanofiber and film formulations, but nanofibers showed to be more flexible even without the addition of plasticizers. In addition to greater hydrophilicity and swelling, nanofibers provided twice as much mucoadhesion than polymer films. *In vitro* drug release studies (potassium hydrogen phthalate buffer, pH 5.46) showed that 30% of the drug loaded in the nanofiber matrix was released within 5 min by a burst release mechanism, achieving 100% within 2 h. The polymer films, in turn, released about 60% of the drug within 30 min, achieving 100% close to 40 min. Therefore, both systems resulted in a rapid drug release. Nanofibers showed a higher antifungal effect than vaginal films. Both polymer nanofibers and films inhibited the *C. albicans* and *C. dubliniensis* growth at 125 and 250 $\mu\text{g}/\text{mL}$, respectively. For *C. albicans*, minimum fungicidal concentration (MFC) values were 125 and 250 $\mu\text{g}/\text{mL}$ for vaginal nanofibers and films, respectively. For *C. dubliniensis*, vaginal nanofibers and film formulations

presented MFC values of 250 and 500 $\mu\text{g/mL}$, respectively. In summary, nanofibers showed to be more advantageous than polymer films in study; however, these results should be confirmed by considering *in vivo* assays (20).

Table I. Novel solid drug delivery systems developed for antifungal agents in vulvovaginal candidiasis.

Formulation type	Composition	Preparation technique	Release profile	Anti-Candida activity	Reference
Intravaginal ring	Polyurethane, castor oil and 10% (w/w) clotrimazole	Fused deposition modeling and 3D printing	A sustained release of the drug during seven days. Cumulative release of 13.4% and 40.1% in simulated vaginal fluid and hydroethanolic solution, respectively.	In agar-diffusion assays, provided an area of growth inhibition of 2 cm. <i>In vitro</i> time-kill analysis displayed a complete growth inhibition of <i>C. albicans</i> after 5 days.	(65)
	Pods: <i>Lactobacillus gasseri</i> blended with sodium carboxymethyl cellulose (25% w/w) and coated with poly(<i>D,L</i> -lactide) (5% w/v) Polymer rings: polydimethylsiloxane	Liquid silicone resin injection molding process	Controlled release for 21 days after a single administration. Release between $1.1\text{--}14 \times 10^7$ cells per day.	Not tested.	(66)
Vaginal films	Carboxymethyl derivative of fenugreek gum, glycerol and nystatin (100,000 units per 1 cm ² film)	Solvent casting method	Fast drug release profile. All the incorporated drug was released within 5 h.	In <i>in vivo</i> studies, the count of <i>C. albicans</i> was reduced from 10^8 to 3×10^2 CFU/mL over 14 days.	(72)
	Tamarind seed polysaccharide, propylene glycol, Tween® 60 and nystatin (10,000 units per 1 cm ² film)		The polymer film released 73.92% of nystatin up to 8 h.	An inhibition halo of 1.1 and 1.5 cm was observed for the film formulation and nystatin solution (control) against <i>C. albicans</i> , respectively.	(76)
	HPMC, chitosan, polyethylene glycol 400 and tioconazole 15% (w/w)		All polymer films showed a fast release of tioconazole in distilled water (almost 100% in 60 min).	Film formulation showed greater activity against <i>C. albicans</i> (inhibition halos between 26 and 34 mm) than both ovule and free tioconazole after 96 h assay.	(19)
	Chitosan, Carbopol® P71NF, polyethylene glycol 400, sorbitol and econazole nitrate 1% (w/w)		An initial burst effect followed by a slow diffusion (30% of drug release after 6 h).	The <i>in vitro</i> activity of the films against <i>C. krusei</i> and <i>C. parapsilosis</i> was twice greater than the commercial cream.	(77)
	Gelucire®, gelatin, genipin and econazole 10% (w/w)		All films controlled the drug release up to 24 h (55-70%). Rapid drug release in the first 5 h (40%) for non-crosslinked systems (without genipin).	Gelucire®-films displayed greater activity against <i>C. albicans</i> than gelatin films alone and films with genipin.	(78)
	HPC, sodium alginate, isopropyl myristate, propylene glycol and clotrimazole (11,11 mg/cm ²)		The optimal film showed a fast release, with 70 and 83% of clotrimazole after 1 and 6 h, respectively.	The inhibition halo against <i>C. albicans</i> for the optimal polymer film was 24 mm and marketed tablet was 23 mm.	(79)

	Chitosan and pectin (75:25), glycerol, dimethyl sulfoxide and fluconazole (16,66 mg/cm ²)	Solvent casting method	The developed vaginal films showed a greater drug release control than the marketed gel. The developed formulation released 55.6 and 79% after 6 and 24 h, respectively. The marketed gel released 74.5 and 77% in the same period.	The <i>in vitro</i> inhibitory activity against <i>C. albicans</i> showed no significant difference between the film and gel formulation.	(80)
	HPMC, polyethylene glycol 400 and fluconazole (68,75 mg/cm ²)		Polymer concentration dependent release (66.5-99.94% after 24 h) Formulation with 3% and 4% of HPMC were more effective in controlling the drug release than polymer films prepared with 1 or 2% of this polymer.	Not tested.	(81)
	Dextran, alginate, polyvinyl alcohol, propylene glycol and clotrimazole 1% (w/w)		Vaginal films released approximately 60% of the drug in 30 min and 98% close to 40 min.	Formulation inhibited the <i>C. albicans</i> and <i>C. dubliniensis</i> growth with a MIC of 250 µg/mL. The MFC was 250 and 500 µg/mL for <i>C. albicans</i> and <i>C. dubliniensis</i> , respectively.	(20)
Vaginal sponges	Chitosan, gelatin, HPMC, Carbopol® 934, calcium chloride, sodium carboxymethylcellulose Tween® 80, butoconazole nitrate (100 mg per sponge)	Freeze drying	Showed a burst release in the first 30 min, with complete release of drug in 24 h. The addition of calcium chloride resulted in a slower initial drug release.	The MIC against <i>C. albicans</i> of vaginal sponges was 4.45 µg/mL (preserved the antimicrobial activity of the drug).	(86)
	Tea tree oil liposomes: soybean phosphatidylcholine, cholesterol, Tween® 80 and tea tree oil 1% (v/v) Sponges: chitosan and glycerol		Not tested	The killing log values (KLV) of pure chitosan sponges against <i>C. albicans</i> were approximately 1-2 after 12 h whereas developed sponges achieved a KLV of 3.	(85)
	Chitosan ascorbate or sodium hyaluronate/lysine acetate and fluoresceine isothiocyanate (model molecule)		Polymeric sponges based on sodium hyaluronate/lysine acetate released 90% of fluoresceine isothiocyanate after 6 h. Polymeric sponges based on chitosan ascorbate, provided a sustained drug release, achieving 30% in this same time interval.	Not tested	(84)

Polymer nanofibers	Poly(lactic-co-glycolic acid) and amphotericin B 4.53% (w/w)	Electrospinning	The amount of released amphotericin B per day was of 12.5%. A complete drug release was achieved at the end of the eighth day, showing a sustained release.	<i>In vitro</i> tests presented values of MIC ranged from 0.25 to 1.0 mg/mL and MFC of 1.0 to 1.5 mg/mL for different <i>Candida</i> species (<i>C. albicans</i> , <i>C. krusei</i> , <i>C. glabrata</i> , and <i>C. tropicalis</i>). <i>In vivo</i> tests showed a complete remission of the infection after 72 h of a single application of nanofibers.	(54)
	Polyvinyl alcohol and fluconazole (320 mg per nanofiber mat)		An initial burst release of 35.13% of drug in 2 h, followed by a sustained release of 97.8% for up to 6 h.	An inhibition zone of 1.2 and 4.8 cm against <i>C. albicans</i> was found for the pure drug and nanofiber, respectively.	(99)
	Nanofibers loaded with free drug: PVP and benzydamine 0.08% (w/w) Nanofibers loaded with nanoparticles: PVP and chitosan/tripolyphosphate nanoparticles loaded with benzydamine 0.08% (w/w)		After 24 h, the amount of drug released from nanofibers with and without nanoparticles was 53.03 and 109.24%, respectively. The presence of nanoparticles provided a controlled release.	Not tested	(100)
	Dextran, alginate, polyvinyl alcohol and clotrimazole 1% (w/w)		30% of the drug was released within 5 min by a burst release mechanism and 100% within 2 h.	Inhibited the <i>C. albicans</i> and <i>C. dubliniensis</i> growth with a MIC of 125 µg/mL. The MFC was 125 and 250 µg/mL for <i>C. albicans</i> and <i>C. dubliniensis</i> , respectively.	(20)

CFU, colony forming units. HPC, hydroxypropyl cellulose. HPMC, hydroxypropyl methylcellulose. KLV, killing log values. MFC, minimum fungicidal concentration. MIC, minimum inhibitory concentration. PVP, polyvinylpyrrolidone.

Table II. Advantages and disadvantages of advanced solid formulations for vulvovaginal candidiasis.

Solid delivery system	Advantages	Disadvantages/technological limitations
Intravaginal rings	-Allows drug release control over long periods (several days); -Self-administration; -Single application; -No interference in sexual activity; -Relatively good patient compliance; -More effective in cases of microbial resistance (drug is released continuously); -No bioadhesive polymers are needed to retain the formulation in the vaginal cavity.	-Fixed shapes and sizes, which limit drugs that may be incorporated (suitable for potent drugs); -Mechanical properties should be strictly controlled to prevent the formulation from deteriorating or expulsion from the vaginal cavity; -Production process is more complex than in polymeric films; -Lower variety of polymeric materials that can be used (cannot be eroded/need to have specific mechanical properties).
Vaginal films	-Different sizes and thicknesses; -Easy and economical to manufacture; -Self-administration; -Avoid leakage and messiness in vaginal cavity; -Less susceptible to microbial contamination than semisolid formulations; -Stabilize drugs susceptible to degradation in aqueous environments; -Various types of polymers can be included in these systems; -Many production techniques are available.	-Bioadhesive polymers should be included in the formulation for mucosal retention; -A compact film structure can result in a low drug release rate due to the low amount of fluid found in the vaginal cavity -Production parameters should be strictly controlled to avoid batch-to-batch variations; -Film thickness may cause discomfort to the patients; -Enough amount of drug should be released in a low volume of fluid.
Vaginal sponges	-Self-administration; -Higher drug loading capacity and drug release compared to the thin films due to their porous nature and greater surface area; -Higher number of interactions between polymers and mucosa structures may be achieved due to greater surface area, enhancing the bioadhesion.	-Lyophilization, the main production technique, is expensive and time-consuming; -Less stable than traditional films; -Polymers should provide sufficient mechanical properties to maintain the 3D structure of vaginal sponges. -Greater thickness compared to thin films can cause some discomfort to patients.
Vaginal nanofibers	-Self-administration; -High surface area, which increases transmucosal interaction and release of larger amounts of drug in low amounts of vaginal fluid; -Electrospun nanofibers from polymer blends, hybrids or composites may be obtained.	-Electrospinning, the main production technique, is expensive and time-consuming; -Large number of process variables to be controlled (applied electric field, distance between the needle and collector, flow rate, needle diameter and polymer solution viscosity); -Less stable than traditional films; -Difficulty controlling the nanofiber morphology and porosity for product uniformity; -Residual organic solvents can be toxic.

6. Final considerations

The emergence of resistant strains of *Candida* requires not only the investigation of new therapeutic options but also the development of advanced formulation. Limitations such as leakage of the conventional semisolid systems and low residence time in the vaginal cavity may be overcome with the solid drug delivery systems presented here. These systems are also more chemically and microbiologically stable and can provide the release of a specific amount of drug to the site of action for a longer period. Vaginal rings allow a drug delivery for several days, reducing the frequency of application and fungal resistance problems. On the other hand, these systems require the inclusion of potent antimicrobial drugs as they are typically designed to act for longer periods and present limited dimensions. Polymer sponges and nanofibers, in turn, may be considered for less potent drugs as greater amounts of drug can be incorporated in their structures and are administered more frequently. Due to the more porous structure and larger surface area, these formulations can provide a greater release of drug in a shorter time (desirable for less potent drugs) and increase bioadhesion (increased surface area results in more interaction with a mucosa). The amount of drug released from each system should be strictly controlled to provide a concentration of drug sufficient for the therapeutic effect. Formulation parameters such as polymer ratio and type, presence of disintegrating and solubilizing agents and polymer combinations may be adjusted within this context. The addition of nanoparticles in these systems affects the mechanical properties and reduces the drug release rate as mentioned in studies presented in this review. Polymer film dimensions may also be modified to achieve a specific release profile. Another aspect that deserves attention in future studies involving the development of solid formulations for VVC is the amount of fluid used in *in vitro* release assays. Literature studies have simulated the release behavior of drugs from the formulation in large amounts of fluid or other solutions, which should be revised to obtain more realistic information. Among the systems selected for this review, more studies were found for thin film formulations. When compared to the other solid systems presented, the production of polymeric films is cheaper and requires fewer unit operations. Although *in vitro* studies performed with different *Candida* strains suggest a significant biological activity for all tested dosage forms, few *in vivo* studies were performed until now and no human studies with these formulations were found.

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Declaration of Competing Interest

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Abbreviation list

AMPs, antimicrobial peptides. BN, butoconazole nitrate. BZY, benzydamine. CA, chitosan ascorbate. CB, Carbopol 971NF. CFU, colony forming units. CH, chitosan. CTZ, clotrimazole. EB, elongation at break. ECN, econazole nitrate. EPS, extracellular polymeric substances. FD4, fluoresceine isothiocyanate dextran. FLZ, fluconazole. HAS, sodium hyaluronate/lysine acetate. HPC, hydroxypropyl cellulose. HPMC, hydroxypropyl methylcellulose. IPM, isopropyl myristate. IVRs, intravaginal rings. KLV, killing log values. MFC, minimum fungicidal concentration. MIC, minimum inhibitory concentration. NP, nanoparticle. PEG, polyethylene glycol. PG, propylene glycol. PLGA, poly(lactic-co-glycolic acid). PN, polymeric nanofibers. PVA, polyvinyl alcohol. PVP, polyvinylpyrrolidone. RVVC, recurrent vulvovaginal candidiasis. SAP, secreted aspartyl proteinase. SD, solid dispersions. SEM, scanning electron microscopy. SVF, simulated vaginal fluid. TCZ, tioconazole. TPP, tripolyphosphate. TPU, thermoplastic polyurethane. TS, tensile strength. TTO, tea tree oil. VVC, vulvovaginal candidiasis.

5 CAPÍTULO III – ARTIGO A SER SUBMETIDO PARA PUBLICAÇÃO

Este capítulo trata do desenvolvimento e caracterização de filmes vaginais a base de biopolímeros (quitosana, pectina e acetato succinato de hidroxipropilmetilcelulose) para a incorporação de ativos antifúngicos (fluconazol e timol) destinados ao tratamento da candidíase vaginal resistente. Biopolímeros foram selecionados tendo em vista que a mucosa vaginal é mais sensível e susceptível a irritação que outros tecidos corpóreos. A quitosana tem sido muito utilizada em formulações vaginais devido as suas propriedades mucoadesivas, porém, pode resultar em filmes quebradiços ou com um perfil de liberação indesejável. Por este motivo, associações de biopolímeros foram estudadas.

O objetivo deste capítulo foi comparar o efeito de combinações poliméricas nas propriedades dos filmes e se a associação de fluconazol e timol é efetiva frente a cepas resistentes de *Candida*. Ainda, um novo método de solubilização da quitosana foi testado. Ao invés da solubilização em ácido acético, que aparece na maior parte dos estudos da literatura, testou-se uma combinação de ácidos cítrico e láctico.

Os filmes vaginais foram desenvolvidos pelo método de evaporação do solvente e então caracterizados através de análises térmicas, espectroscópicas, cristalográficas, morfológicas e mecânicas. Para entender o comportamento dos filmes após contato com o fluido vaginal, ensaios de molhabilidade e de intumescimento foram considerados. A permeabilidade do fluconazol a partir dos filmes mais promissores foi avaliada através do modelo da câmara de Franz e mucosa vaginal suína. O efeito destas formulações na desestruturação de domínios lipídicos e proteicos da mucosa foi avaliado por ATR-FTIR. Por fim, avaliou-se a atividade da associação de fluconazol e timol frente a *C. glabrata* (cepa resistente de *Candida*) pelo ensaio de difusão em placa.

O produto deste capítulo é um artigo científico, que será submetido para apreciação na revista *Carbohydrate Polymers*.

5.1 ARTIGO

**BIOPOLYMER COMPOSITE FILMS BASED ON CHITOSAN FOR
RESISTANT VAGINAL CANDIDIASIS**

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ABSTRACT

Vulvovaginal candidiasis (VVC) is an opportunistic infection caused by a fungus of the *Candida* genus, affecting approximately 75% of women during their lifetime. Fungal resistance cases and adverse effects have been the main challenges of oral therapies. In this study, the topical application of thin films containing fluconazole (FLU) and thymol (THY) is purposed to overcome these problems. Vaginal films based only on chitosan (CH) or combining this biopolymer with pectin (PEC) or hydroxypropylmethylcellulose acetate succinate (HPMCAS) were developed by the solvent casting method. In addition to a higher swelling index, CH/HPMCAS films showed to be more plastic and flexible than systems prepared with CH/PEC or only chitosan. Biopolymers and FLU were found in an amorphous state, contributing to explaining the rapid gel formation after contact with vaginal fluid. High permeability rates of FLU were also found after its immobilization into thin films. The presence of THY into polymer films increased the distribution of FLU in vaginal tissues and resulted in an enhanced anti-*Candida* activity. A significant activity against the resistant *C. glabrata* was achieved, reducing the required FLU dose by 50%. These results suggest that the developed polymer films represent a promising alternative for the treatment of resistant vulvovaginal candidiasis, encouraging clinical trials.

Keywords: Vaginal candidiasis; polymeric films; fluconazole; chitosan; pectin; hydroxypropylmethylcellulose acetate succinate.

1 INTRODUCTION

Vulvovaginal candidiasis (VVC) is a fungal infection caused by *Candida albicans* and non-*albicans* species such as *C. tropicalis*, *C. glabrata* and *C. parapsilosis* (Pfaller & Diekema, 2007). This pathology involves an abnormal growth of yeasts in the vaginal mucosa and represents one of the most common diseases in women seeking gynecological care, affecting approximately 75% of this population (CDC, 2023). Every year, a global incidence of 138 million new recurrent VVC cases is estimated, in which the patient has at least four confirmed episodes of the disease in a year (Denning et al., 2018).

VVC treatment is often performed with the topical or oral administration ofazole antifungal drugs (e.g., miconazole, fluconazole, itraconazole) or then polyenes such as the nystatin and amphotericin B. Although effective in most cases, the oral administration of azoles is characterized by extensive hepatic metabolism and can result in side effects such as headaches, cardiomyopathy and gastrointestinal disturbances (Nsenga & Bongomin, 2022). Oral treatment is also contraindicated for pregnant women, which is a public in that high recurrence of VVC is found (Mølgaard-Nielsen et al., 2016). For these reasons, the oral administration has been replaced by the local application. In addition to the ease of application, other advantages that could be achieved include a sustained drug release, improve of patient adherence to treatment and increased efficacy (Machado et al., 2015).

The introduction of anti-*Candida* treatment without confirmation of the etiologic agent, prophylactic use and long-term therapies also contribute to the emergence of resistant strains (Gilhotra et al., 2014). In addition to overexpression of efflux pump genes, 3D structures known as “biofilms” can be found, which restrict the penetration of immune system cells and antifungal drugs (Palmeira-de-Oliveira et al., 2015). Therefore, an increased dose to obtain the same efficacy (which increase the occurrence of adverse effects) or then the association of drugs acting by different mechanisms is required to overcome this problem (Soares et al., 2018).

Essential oils (EOs) and their constituents emerge as an alternative to cases of resistance in a series of studies, which are often combined with azoles (Jafri & Ahmad, 2020; Palmeira-de-Oliveira et al., 2013). In addition to an antimicrobial activity, they are characterized by low toxicity, which makes them an attractive therapeutic option (Palmeira-de-Oliveira et al., 2015). Jafri & Ahmad (2020), in turn, demonstrated that

the EO of the *Thymus vulgaris* species can act synergistically with fluconazole against *C. tropicalis*. Thymol, the major constituent of this EO, shows a synergistic action against *C. albicans* as well as *in vitro* inhibition of biofilm formation.

Commercially available topical formulations such as creams and ointments are characterized by low adhesion to the vaginal mucosa, which lead to leakage from the vagina cavity as well as low dose uniformity (Johal et al., 2016). Solid dosage forms, in turn, have been prioritized as they result in a more controlled drug release, are more stable to degradation and oxidation, have a low water content and are patient friendly systems as no leaking in the vaginal canal is observed. In recent years, polymer films have been extensively studied and developed for this purpose (Calvo et al., 2019; Conte et al., 2023).

Chitosan (CH) is a natural polysaccharide extensively used as a polymeric matrix in polymer films (Sanap et al., 2022). It is a weak base ($pK_a = 6.51$) that demands an acid medium for its complete solubilization. Due to the presence of amino and hydroxyl groups in its chemical structure, it can be linked to mucin of vaginal mucus, providing a lasting mucoadhesive effect (Pelegrino & Seabra, 2017). Despite the high biocompatibility, chitosan films have limited mechanical properties (high hardness). Therefore, plasticizing agents or other polymers should be included in the film composition (Riccio et al., 2022) or even polymer derivatives can be synthesized.

Polymer mixtures are able to improve the mechanical properties as well as the drug release profile (Bartczak & Galeski, 2014). The main challenge during the formulation design is precisely to select polymers compatible among them (Neves & Sarmiento, 2017). The combination of biopolymers has been prioritized in drug delivery platforms targeted to vaginal administration due to greater biodegradability and biocompatibility (Kolakovic et al., 2012).

In view of all the issues mentioned above, this study aimed to develop biopolymer films containing fluconazole (FLU) and thymol (THY) for the treatment of resistant vulvovaginal candidiasis. Given that comparative studies among different biopolymer blends are scarce in the literature, chitosan, pectin and hydroxypropylmethyl cellulose acetate succinate (HPMCAS) biopolymers were selected due to the high biocompatibility and film-forming properties.

HPMCAS is a semi-synthetic polymer derived from cellulose, which is able to dissolve in the presence of vaginal fluid (Notario-Pérez et al., 2019). Pectin, in turn, is a polysaccharide formed by monomers of galacturonic acid, which are linked together by

glycosidic bonds. When cross-linked with chitosan, an increased residence time at the site of action is expected (Lazaridou & Biliaderis, 2020; Mishra et al., 2017).

The thin films were initially prepared by solvent casting method and then characterized in terms of thermal, morphological, spectroscopic, mechanical, wettability and swelling properties. The vaginal permeability of fluconazole immobilized into different polymeric matrices was evaluated in Franz-type diffusion cells and porcine vaginal mucosa. Finally, *in vitro* antifungal activity assays were carried out to confirm the benefits of the combination between FLU and THY. Another novelty of this study was the solubilization method of chitosan. Most literature studies solubilize the polymer in acetic acid, but a combination of citric and lactic acids was tested here.

2 MATERIALS AND METHODS

2.1 Materials

Fluconazole (99% purity) and thymol (98.5% purity) were purchased from Infinity Pharma (Campinas, SP, Brazil) and Sigma-Aldrich (St. Louis, MO, US), respectively. Chitosan (450 KDa and 61% of *N*-deacetylation) and pectin (18.29% of esterification) were purchased from Sigma-Aldrich whereas HPMCAS from the Ashland (Jaguaré, SP, Brazil). Citric acid and lactic acid were obtained from Sigma-Aldrich and VETEC (Duque de Caxias, RJ, Brazil), respectively. Methanol (HPLC grade) and acetonitrile (HPLC grade) were purchased from Merck (Darmstadt, Germany). Water was obtained from a Milli-Q® water purification system (Millipore, Milford, MA, US).

Simulated vaginal fluid (SVF) was prepared by considering 3.51 g NaCl, 1.40 g KOH, 0.222 g Ca(OH)₂, 0.018 g bovine serum albumin, 2 mL lactic acid, 1 mL acetic acid, 0.16 g glycerol, 0.4 g urea and 5.0 g glucose for 1 L of distilled water. Phosphate buffered (PBS) saline was prepared with 10.1 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 136.9 mM NaCl, 2.7 mM KCl and adjusted to pH 7.4 with 1 M HCl. Krebs-Ringer bicarbonate buffer was prepared with 119 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, 2.5 mM CaCl₂·2H₂O, 1.0 mM MgCl₂·6H₂O, 5.5 mM glucose and adjusted to pH 7.4 with 1 M HCl. All other chemical reagents and solvents used for the analysis were of analytical grade.

2.2 Methods

2.2.1 Preparation of vaginal films

Film formulations were prepared by the solvent casting technique. In an initial screening, CH was solubilized in several acid types for selecting the formulations with improved characteristics. CH films showed to be brittle or then fluconazole crystallized depending on type of acid considered. For CH films prepared only with citric acid, low adhesiveness was found. Therefore, low concentrations of lactic acid (0.25% v/v) were combined with citric acid (3% w/v) to obtain more flexible and adhesive polymer films, which is crucial properties for application in vaginal mucosa. After the solubilization of 1% (w/v) CH in this acid combination, the pH value of final solution was 4.09.

HPMCAS (1% w/v) and PEC (1% w/v) solutions were prepared separately in water and stirred overnight. CH:HPMCAS or CH:PEC solutions were mixed in a ratio of 12:1. This proportion was defined with the objective of obtaining formulations with ideal malleability and mucosal adhesiveness. FLU (0.6% w/v) and THY (0.6% w/v) were solubilized into 2 mL of ethanol and then added to each polymer solution under stirring until complete dissolution. Blank polymer films (without FLU and THY) were developed following the same procedure. The final solutions (65 mL) were poured in Teflon plates with area of 45.36 cm² and then dried at 30 °C for 4 days. The formulations were stored in a humidity-controlled desiccator containing dried silica gel to prevent the absorption of water. A detailed description of the composition of films is presented in Table 1.

Table 1. Composition of biopolymer films for vaginal infections.

Film formulation	Chitosan (%)	Constituents			
		Pectin (%)	HPMCAS (%)	Fluconazole (%)	Thymol (%)
FI	1				
FII	1			0.6	
FIII	1			0.6	0.6
FIV	1	1			
FV	1	1		0.6	
FVI	1	1		0.6	0.6
FVII	1		1		
FVIII	1		1	0.6	
FIX	1		1	0.6	0.6

2.2.2 Differential scanning calorimetry (DSC)

DSC assays were performed using a TA Instrument Q200 calorimeter (New Castle, US). Samples of each film (6-10 mg) were placed in aluminum pans, and the thermal properties were monitored at a heating rate of $10\text{ }^{\circ}\text{C min}^{-1}$ under a constant nitrogen atmosphere (flow of 50 mL min^{-1}). The samples were heated from -10 to $270\text{ }^{\circ}\text{C}$. Pure CH, PEC and HPMCAS were heated isothermally at $110\text{ }^{\circ}\text{C}$ for 3 min to eliminate the water, cooled using liquid nitrogen and then reheated to $270\text{ }^{\circ}\text{C}$.

2.2.3 Thermogravimetric analysis (TG)

Thermogravimetric (TG) analyses were performed on a TGA-50 equipment (Shimadzu, Kyoto, Japan). Approximately 15 mg of each polymer film was placed into a platinum pan. The thermograms were recorded in the range of 20 - $800\text{ }^{\circ}\text{C}$, with a heating rate of $10\text{ }^{\circ}\text{C min}^{-1}$, under a nitrogen atmosphere (50 mL min^{-1}). Temperature ranges from DrTG curves allow to identify thermal decomposition or thermo-oxidative degradation.

2.2.4 Fourier-transform infrared spectroscopy (FT-IR)

Samples of raw materials (CH, PEC, HPMCAS, FLU, THY, citric acid and lactic acid) were mixed with KBr in a ratio of 1:15 and the discs were prepared by using a hydraulic press. IR spectra (4000 - 400 cm^{-1}) were taken from IRPrestige 21 equipment (Shimadzu, Kyoto, Japan) in absorbance mode, at room temperature, with 25 scans and a resolution of 2 cm^{-1} .

For the developed formulations (FI – FIX), FT-IR spectra were obtained in the Attenuated Total Reflection (ATR) mode by considering a frequency range from $4,000$ to 700 cm^{-1} , at room conditions, with the collection of 25 scans at a resolution of 2 cm^{-1} .

2.2.5 X-ray diffraction (XRD)

X-ray diffraction measurements were performed on a Bruker model D2 Phaser instrument (Karlsruhe, Germany) using $\text{K}\alpha$ copper radiation ($\lambda=1.5418\text{ \AA}$) and LynxEye unidimensional scintillation detector. The X-ray generator was set to 30 kV and 10 mA . Samples were scanned in the range 5 - $40^{\circ} 2\theta$, with an angular step of $0.091^{\circ}\text{ s}^{-1}$.

2.2.6 Thickness, tensile strength and percentile elongation at break

The thickness of five rectangular specimens was first determined by the average in three different film regions. Film formulations (90 × 12.5 mm) were stored for 24 h at room temperature (25 ± 2 °C) and $58 \pm 5\%$ of relative humidity before evaluating the mechanical properties. The tensile properties of prepared materials were measured using a universal tensile testing machine with a load cell of 50 N (EMIC – DL200, São José dos Pinhais, PR, Brazil). The gauge length and the crosshead speed were 50 mm and 10 mm min⁻¹, respectively.

The values of Young's modulus, tensile strength and elongation at break were calculated from the stress-strain curves. Tensile strength is the force per unit cross-sectional area required to break the polymer film. The load at failure is recorded and the tensile strength is calculated. The percentage elongation of the film during the assay allows the calculation of elongation at break, which indicates the plasticity of the film. Young's modulus or modulus of elasticity is the slope of the linear part of the curve and toughness represents the area under the stress-strain curve. All these parameters are affected by the polymer blend of the film and plasticizing agents (Machado et al., 2013).

2.2.7 Wettability

The contact angle in the solid (film)/liquid interface was determined by using a Ramé-Hart model 250-F1 goniometer (Succasunna, US). For the measurements, 3 µL of distilled water was deposited on the film surface. The dynamic contact angle was evaluated in intervals of 10 s. Ten measures were performed for each film formulation and analyzed in DropImage version 3.54 software (Ramé-Hart Instrument co., Succasunna, NJ, US). All analyzes were performed in triplicate.

2.2.8 Swelling behavior

Circular sections of formulations presenting 1.77 cm² were weighted (W_0) and placed on beakers containing 10 mL of simulated vaginal fluid (pH 4.2) at 37 °C. The films were taken from beakers at different intervals (1, 5, 10, 20, 30 and 60 min) and then weighted (W_t). The swelling index (SI) of the films was then calculated by using the following equation:

$$SI(\%) = \frac{(W_t - W_0)}{W_0} \times 100$$

2.2.9 Scanning electron microscopy (SEM)

SEM analyses were performed to evaluate surface and fracture morphology of the formulations. Upper side and fracture of films were fixed on stubs with double adhesive carbon tape. Fractures were created after treatment of formulations with liquid nitrogen. All samples were coated with gold palladium using a gold sputter module in a high vacuum evaporator. Samples were then observed in an JSM-6390LV Scanning Electron Microscope (JEOL, Tokyo, Japan) selecting an acceleration voltage of 10 kV.

2.2.10 *Ex vivo* permeability studies

2.2.10.1 *Preparation of vaginal tissue samples*

Freshly excised porcine vaginal tissue was obtained from a local abattoir immediately after slaughter and transported in cold Krebs buffer (pH 7.4). Manual separation of the intact vaginal mucosa was performed using surgical scissors and scalpel.

2.2.10.2 *Permeability and retention through the vaginal mucosa*

After the preparation of biological tissues, they were immediately clamped between the donor and receptor chambers of Franz-type diffusion cells presenting a diffusional area of 1.77 cm². The receptor chambers were filled with 10 mL of phosphate-buffer saline pH 7.4 (PBS) and kept at 37 °C under constant magnetic stirring at 280 rpm. The formulations FII, FIII, FVI and FIX were cut into 6 mm disks (equal to 2.2 mg of FLU) and applied in the vaginal mucosa with 200 µL of simulated vaginal fluid (SVF). An aqueous solution of FLU (2 mL at 1.1 mg mL⁻¹) was used as control.

At time intervals of 1, 2, 3, 4, 5, 6 and 7 h, 400 µL of the receptor fluid were removed, with fresh buffer replacement, to evaluate the amount FLU permeated by high-performance liquid chromatography (HPLC) (method described below). At the end of the permeation assay, the amount of FLU retained in the vaginal mucosa was also analyzed. The tissue was cleaned using a cotton swab embedded with Milli-Q water and cut in small pieces. The extraction of FLU from the mucosa was performed using 3 mL

of an acetonitrile/Milli-Q water mixture (2:1), followed by overnight storage to increase the extraction efficiency. After this period, the tissues were sonicated for 30 min and centrifuged at 6,200 rpm for 30 min to precipitate proteins. The supernatant was filtered and then injected into HPLC.

2.2.10.3 HPLC analysis

Quantification of FLU from permeability studies was performed by HPLC. The samples were analyzed using a Perkin-Elmer Series 200 HPLC system (Waltham, MA, US) equipped with a UV detector, pump, autoinjector and online degasser. The chromatographic separation of FLU was performed on a C18 column (250 x 4.6 mm, 5 μ m particle size; Perkin Elmer, Shelton, US) preceded by a guard column of the same packing material (KJ0-4282, Phenomenex®, Torrance, CA, US) at room temperature. The mobile phase was composed of water and methanol in a ratio of 60:40 v/v. Elution was performed isocratically at a flow rate of 1 mL min⁻¹ with a sample injection volume of 20 μ L and a total sample acquisition time of 11 min. The wavelength of the detector was set to 261 nm. The method was specific and linear in the range of 0.6 to 100 μ g mL⁻¹ ($r^2 > 0.99$). No interference of biological matrix on the drug detection was observed.

2.2.10.4 Calculations of the permeability data

The cumulative amount of drug permeated (Q_t) was calculated by the following equation:

$$Q_t = V_r C_t + \sum_{i=0}^{t-1} V_s C_i$$

where V_r and V_s represent the receptor solution and sample volumes; C_t is the drug concentration on the receptor medium at each sampling time and C_i is the accumulated drug concentration at the i th time. Data were expressed by Q_t/S ($S = 1.77 \text{ cm}^2$), the cumulative amount of drug permeated mucosal area per unit.

The steady-state flux (J_{ss}) was calculated from the linear slope of the cumulative amount of FLU permeated *versus* time. The permeability coefficient (P) was determined by the relation between J_{ss} and the initial donor concentration (C_d). Lag time was

expressed as the time required to achieve the J_{ss} , which is represented by the intercept of the regression line with the abscissa.

2.2.11 ATR-FTIR analysis of the vaginal mucosa

The vaginal mucosa was carefully separated as already described. Tissues were treated with PBS or film formulations during the same period of permeation assay (7 h). ATR-FTIR spectra were obtained in a frequency range from 600-4,000 cm^{-1} on Perkin-Elmer Frontier equipment (Waltham, US) with the collection of 16 scans at a resolution of 4 cm^{-1} .

2.2.12 Antifungal activity

The *in vitro* antifungal activity of the films contained FLU and THY was evaluated by disk diffusion method ($n=3$), with modifications, according to the M44-A2 protocol (CLSI 2009). A formulation prepared with CH 1% and without drug was used as control. The assay was performed on Sabouraud Dextrose Agar (SDA) plate considering *C. albicans* (ATCC 10231), *C. krusei* (ATCC 6258), *C. parapsilosis* (ATCC 22019) and *C. glabrata* (CG MT 29) strains. All strains were donated from the yeast mycology collection of the Federal University of Rio Grande do Sul (UFRGS).

The SDA plate surface was inoculated with each yeast suspension adjusted at 0.5 McFarland, and containing approximately $1-5 \times 10^3$ CFU/mL, in sterile 0.9% saline solution. The formulation films were cut into 6 mm disks and laid down on the agar plate, which were incubated at 37°C for 48 h. The films antifungal activity on the plate was determined according to the size of inhibition halo.

2.2.13 Statistical analysis

Results were analyzed using GraphPad Prism software (version 6.0). One-way ANOVA followed by Tukey's *post-hoc* test was used for multiple comparisons.

3 RESULTS AND DISCUSSION

3.1 Thickness and macroscopic analysis

Similar macroscopic features were found for the polymer films, which showed a yellowish color. The low standard deviation in the average thickness suggests that the developed film formulations are uniform (Table 2). The average thickness of films was smaller than that of Dobaria et al. (2009) (0.46 mm) and close to that of Calvo et al. (2019) (~0.25 mm). These thickness values are compatible with a vaginal application, avoiding discomforts for the patients.

For each polymer blend or chitosan alone (same column), no statistical difference was observed in this parameter after incorporation of FLU or FLU/THY (ANOVA/Tukey's test). On the other hand, formulations prepared only with the CH (FI, FII and FIII) showed thickness values statistically different from formulations combining this polymer with PEC and HPMCAS. Similar findings were found in a literature study comparing CH and CH/PEC-based films (Fu et al., 2022). No significant difference in this parameter was observed between CH/PEC and CH/HPMCAS films. The electrostatic interaction between CH (positive charge) and PEC or HPMCAS polymers (negative charge) and other types of chemical interactions between the polymers such as hydrogen bonding and Van der Waals forces could lead to a greater molecular interaction, explaining the reduction in film thickness (Sopyan et al., 2022).

Table 2. Thickness values of developed films ($n=3$).

Sample	Thickness (mm)	Sample	Thickness (mm)	Sample	Thickness (mm)
FI	$0.37 \pm 0.02_{(a)}$	FIV	$0.26 \pm 0.03_{(b)}$	FVII	$0.28 \pm 0.01_{(b)}$
FII	$0.40 \pm 0.03_{(a)}$	FV	$0.28 \pm 0.02_{(b)}$	FVIII	$0.30 \pm 0.02_{(b)}$
FIII	$0.39 \pm 0.05_{(a)}$	FVI	$0.30 \pm 0.01_{(b)}$	FIX	$0.31 \pm 0.01_{(b)}$

Data were expressed as mean $\pm$ SD. Different letters indicate statistical difference among formulations ($p < 0.05$).

3.2 Thermal analysis

3.2.1 Thermogravimetric analysis

Thermogravimetry was considered to evaluate the thermal stability of the pure constituents and polymer films (Fig 1; Table S1). The TG and DrTG curves of FLU (Fig. 1A and B) presents an initial weight loss of 1.85% between 70 and 90 °C, which is related to the removal of residual water. A most relevant mass loss was found from 200 °C with almost complete degradation (97.48%), which is attributed to decomposition of monohydrate FLU (Caira et al., 2004). THY, in turn, completely degrades in a single thermal event at 60-188 °C (Cao et al., 2022; Wu et al., 2021).

All polymers presented three main mass loss events (Fig. 1A and B; Table S1). PEC presents an initial mass loss of 15.20%, which is probably associated with the loss of residual water. The second event is characterized by the degradation of the main polymer fraction (51.32%). Finally, the last event (mass loss of 13.1%) is probably related to fractions of the polymeric chain that are stable at lower temperatures and reacted during the analysis at higher temperatures (Mishra et al., 2009). HPMCAS has a mass loss of 1.02% at 20-94 °C attributed to residual water, the major mass loss (88%) at 190-425 °C associated with the polymer degradation and a final event at 537 °C with degradation of 8.43% of HPMCAS (Gökmen & Pekel Bayramgil, 2022). The loss of residual water is also observed for CH (Homez-Jara et al., 2018) as well as other two events related to the degradation of deacetylated (51%) and acetylated fraction (30%).

All polymer films presented a similar thermal behavior (Fig. 1C-H), presenting two main events. The first event occurred between 40 and 310 °C (mass loss of about 55%) and the second thermal event from 310 and 600 °C (mass loss close to 30%). The first degradation event of the films results from the degradation of several components (FLU, THY, citric and lactic acid) and polymer fractions. The second event, in turn, is related to a probable degradation of polymer fractions (CH, PEC and HPMCAS). A drastic reduction in the thermal stability of chitosan is found when films of this polymer are compared to the raw material. On the other hand, the formulations containing only CH maintained the residual mass, indicating a reduction in the degradation temperature and not an increase in the amount of degraded polymer.

All polymer films presented degradation events from 50 to 310 °C. This reduction in thermal stability between pure CH and CH-based films may be related to

interactions between citric acid and CH, which can explain the degradation of all polymer films at approximately 310 °C (common constituent among all formulations). Citric acid, which presented a total degradation up to 306 °C, is the major constituent of the films. No changes in the thermal stability were also observed when another polymer (PEC or HPMCAS) was added to CH-based films.

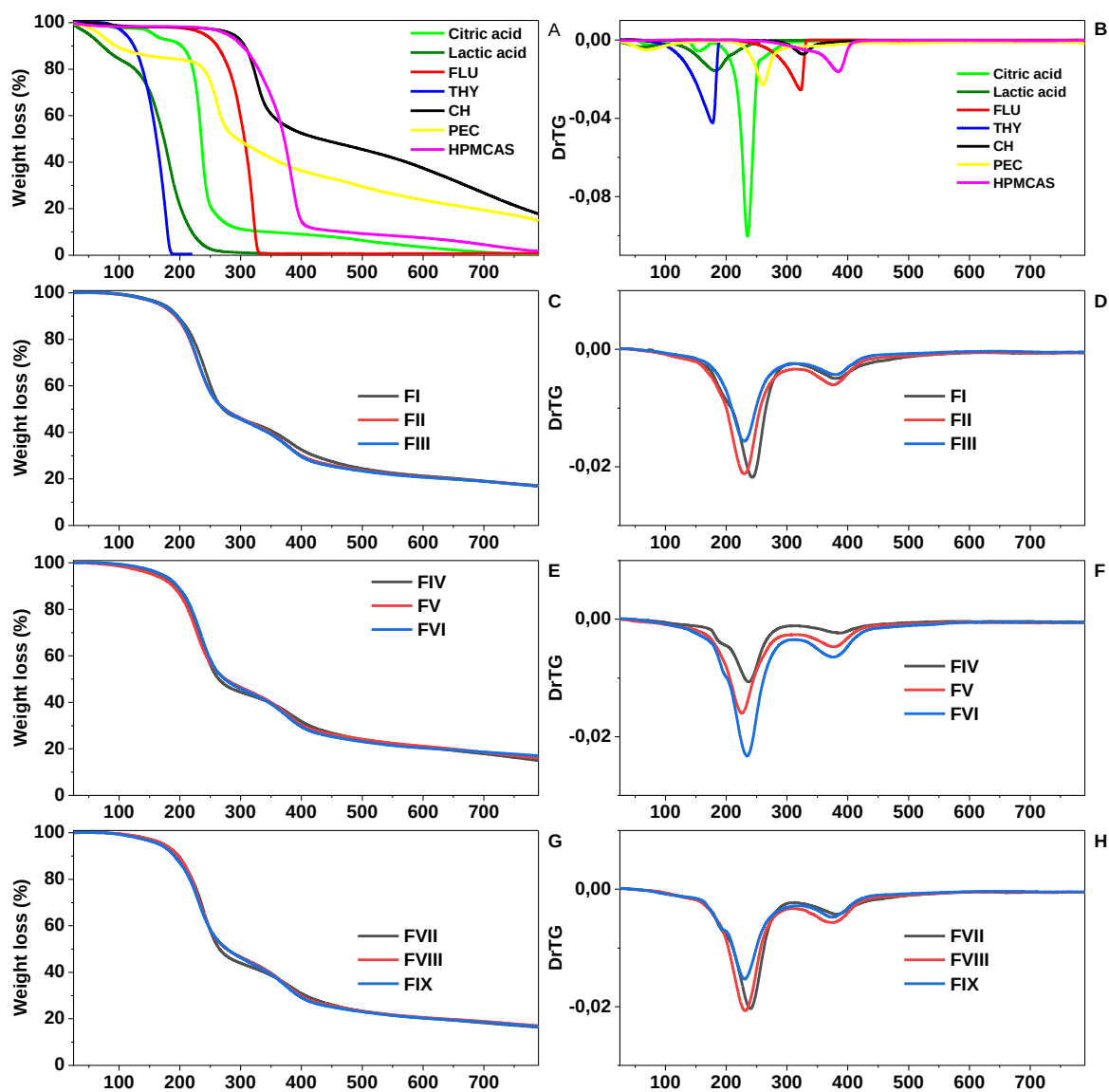


Figure 1. Thermogravimetric analysis of pure constituents (A and B) and polymer films (C, D, E, F, G and H).

3.2.2 Differential scanning calorimetry

The DSC analyses (Fig. 2; Table S2) were performed to investigate possible interactions among constituents of polymer films. Samples were previously stored in a desiccator with silica to ensure a very low relative humidity (Enumo et al., 2020). FLU thermogram showed a sharp endothermic peak at 141.2 °C (120 J g⁻¹), which corresponds to its melting temperature (T_m) (Caira et al., 2004; Sanap et al., 2022; Teleginski et al., 2015). Two thermal events were identified for the THY, its melting at 52 °C and an event at approximately 143 °C. Trivedi et al. (2015) reported an endotherm close to 148 °C for the THY, which was attributed to its oxidation (Trivedi et al., 2015).

For polymers, PEC showed an endotherm at 106 °C (463.2 J g⁻¹) related to its melting, an exotherm at 240.3 °C attributed to its crystallization (T_c) and also an event at 190°C indicating the onset of its degradation (Einhorn-Stoll et al., 2007). The polymer chains of PEC reorganize and release energy before degradation (Mishra et al., 2009). The HPMCAS showed a glass transition temperature (T_g) at 58 °C whereas the CH presented two T_g (70 and 200 °C).

All CH-based films presented similar endotherms between 141 and 198 °C, which probably is related to its melting temperature and interactions of all constituents. No characteristic thermal events of pure polymers were observed, indicating that they interact well with each other. Once the citric acid is found at high concentrations in the polymer films, its degradation event affected the DSC analyses of these formulations, overlaying most thermal events. Endothermic events below 150 °C are related to FLU melting and degradation of acids, THY and polymer fractions, making it difficult to obtain specific conclusions for each film constituent.

No thermal transitions below 141.6 °C were observed in polymer films, suggesting that fluconazole is found in an amorphous form. The combination of polymers also seems to have reduced the crystallinity of the CH films, particularly for the combination PEC/CH. Another point is that polymers and film constituents showed to be compatible with each other.

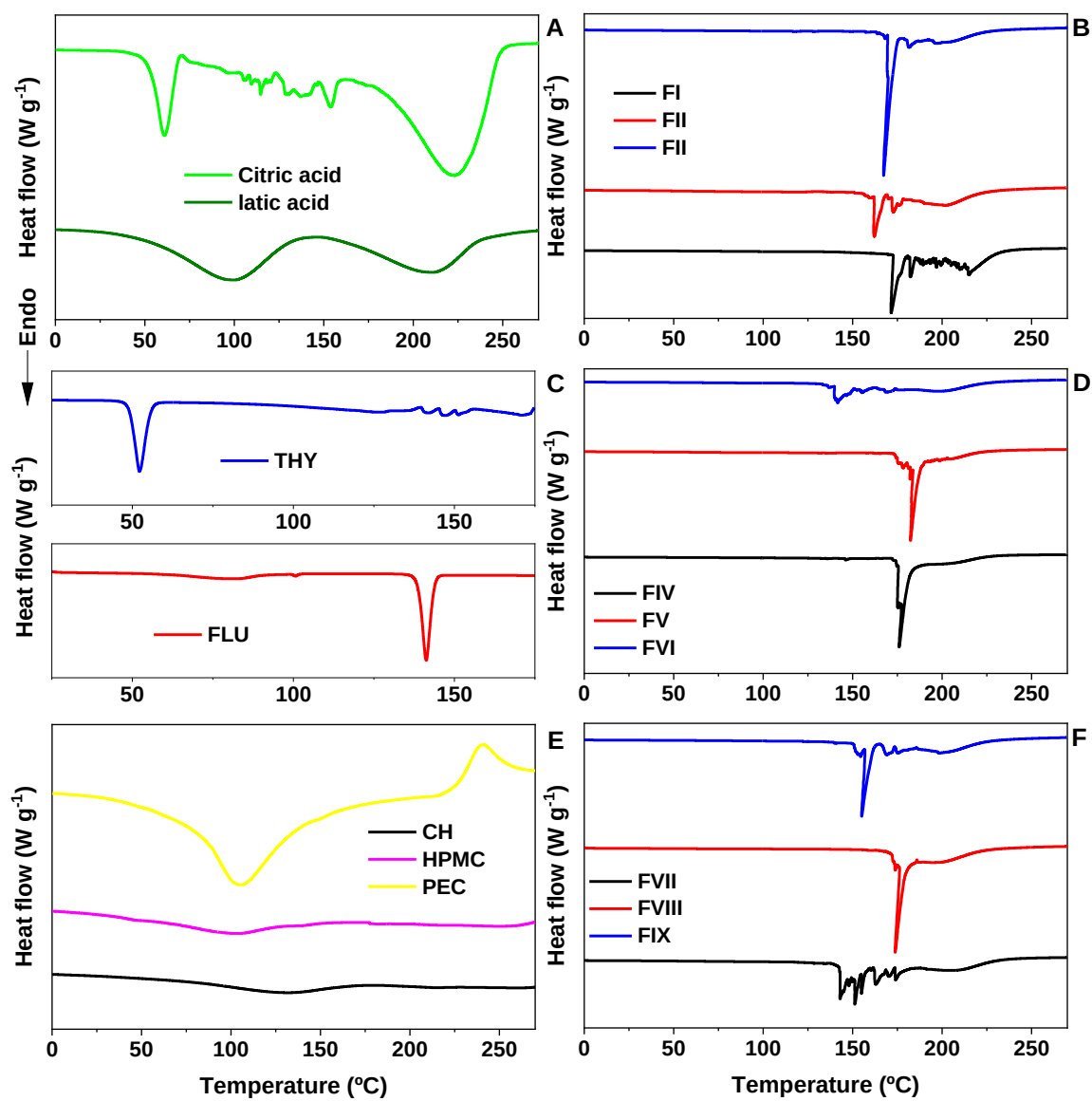


Figure 2. DSC thermograms of acids (A), fluconazole and thymol (C), polymers (E), and films (B, D and F).

3.3 FT-IR spectra

Fig. 3 presents the FT-IR spectra of pure film constituents and film blends. For the FLU, a large band in the region between 3,400 and 2,800 cm^{-1} is observed, which corresponds to O—H stretching from intramolecular and intermolecular hydrogen bonds and C—H stretching vibration. Bands at $\sim 1,607 \text{ cm}^{-1}$ correspond to C=C stretching of aromatic ring and bands in 1,515 cm^{-1} and 1,421 cm^{-1} are related to stretching vibration of triazole rings. C—F stretching vibration can be observed at 1,277 cm^{-1} (Caira et al., 2004; Teleginski et al., 2015). The IR spectra of THY, in turn, shows broad stretching vibrational bands at $\sim 3,230 \text{ cm}^{-1}$ due to O—H groups. C—H stretching vibrations are found at 2,951 and 2,858 cm^{-1} , which are related to CH_3 and CH_2 groups, respectively. In the range $\sim 1,750\text{--}1,250 \text{ cm}^{-1}$, C=C stretching as well as O—H and C—O bending vibrations of phenolic groups are observed. The plane aromatic C—H wagging vibrations are identified at $\sim 809 \text{ cm}^{-1}$ (Wu et al., 2021).

For chitosan, a broad band in the region $\sim 3,400 \text{ cm}^{-1}$ is observed, which corresponds to the stretching vibrations of intramolecular hydrogen bonds in O—H groups, overlapping the stretching band of N—H. The absorption bands at 2,928 and 2,888 cm^{-1} are attributed to C—H symmetric and asymmetric stretching, respectively. Bands at 1,655 and 1,373 cm^{-1} are respectively related to C=O stretching of amide I and C—N stretching of amide III in *N*-acetyl groups. N—H bending in NH_2 is confirmed by the presence of a band in 1,598 cm^{-1} . Finally, the band at 1,050 cm^{-1} corresponds to C—O stretching vibration whereas the band at 898 cm^{-1} corresponds to β -(1,4) glycosidic unions in C—H (Calvo et al., 2019; Silva et al., 2020).

For PEC, the stretching vibrations of O—H and C—H from alkyl groups can be observed at 3,450 and 2,936 cm^{-1} , respectively. The stretching vibration from the carbonyl (C=O) group is identified at 1,639 cm^{-1} . The characteristic absorption band at 1,050 cm^{-1} can be attributed to asymmetric stretching vibration of the C—O—C (Bichara et al., 2016; Mishra et al., 2009).

The HPMCAS spectra also show a broad stretching vibrational band at 3,600–3,200 cm^{-1} attributed to O—H groups and a band at 2,934 cm^{-1} for C—H from methyl groups. The band at 1,736 cm^{-1} is associated with vibrations of ether groups and the band at 1,373 cm^{-1} corresponds to CH_3 bending vibrations. The most intense band in the HPMCAS spectrum occurred at 1,050 cm^{-1} from C—O and represents out-of-phase

vibrations associated with alkyl substituted cyclic ring containing ether linkages. The band at 938 cm^{-1} , in turn, represents the in-phase vibrations from ether linkages and appeared attached to the band at $1,050\text{ cm}^{-1}$ (Calvo et al., 2019; Gökmen & Bayramgil, 2022). The spectrum of citric acid shows more intense bands at $\sim 3,400$, $1,727$ and $1,624\text{ cm}^{-1}$. The first band is attributed to stretching vibration of O—H groups and the last two bands to carbonyl groups (C=O) (Pimpang et al., 2018).

The Figures 3B, D and F show the spectra of developed polymer films (FI – FIX). In spectra FII, FIII, FV, FVI, FVIII and FIX, representative bands of FLU appear in $1,617\text{ cm}^{-1}$ (corresponding to C=C stretching vibration of aromatic ring), $1,517$ and 965 cm^{-1} (triazole rings). The presence of THY in FIII, FVI and FIX is confirmed by bands at $\sim 2,900$ and $1,279\text{ cm}^{-1}$, which correspond to methyl groups of THY. These characteristic bands of fluconazole and thymol suggest an effective incorporation of these compounds in the polymer matrices. Talón et al. (2017) also observed a reduction in band intensity when thyme extract (with thymol as the major compound) was added to CH-based films.

All polymer films showed a main band at $\sim 1,700\text{ cm}^{-1}$ (from C=O stretching vibration), which could be related to citric acid. As already mentioned, this compound is found at higher concentrations in formulations than all other constituents. For PEC/CH films with FLU and thymol, a reduction in band intensity was observed when compared to films prepared only with chitosan or then CH/HPMCAS, suggesting an enhanced interaction between PEC and CH in presence of these agents. When FLU and thymol were not incorporated into PEC/CH films, this inter-polymer interaction was weakened. Overall, the spectroscopic data showed that CH and the other compounds were miscible and compatible.

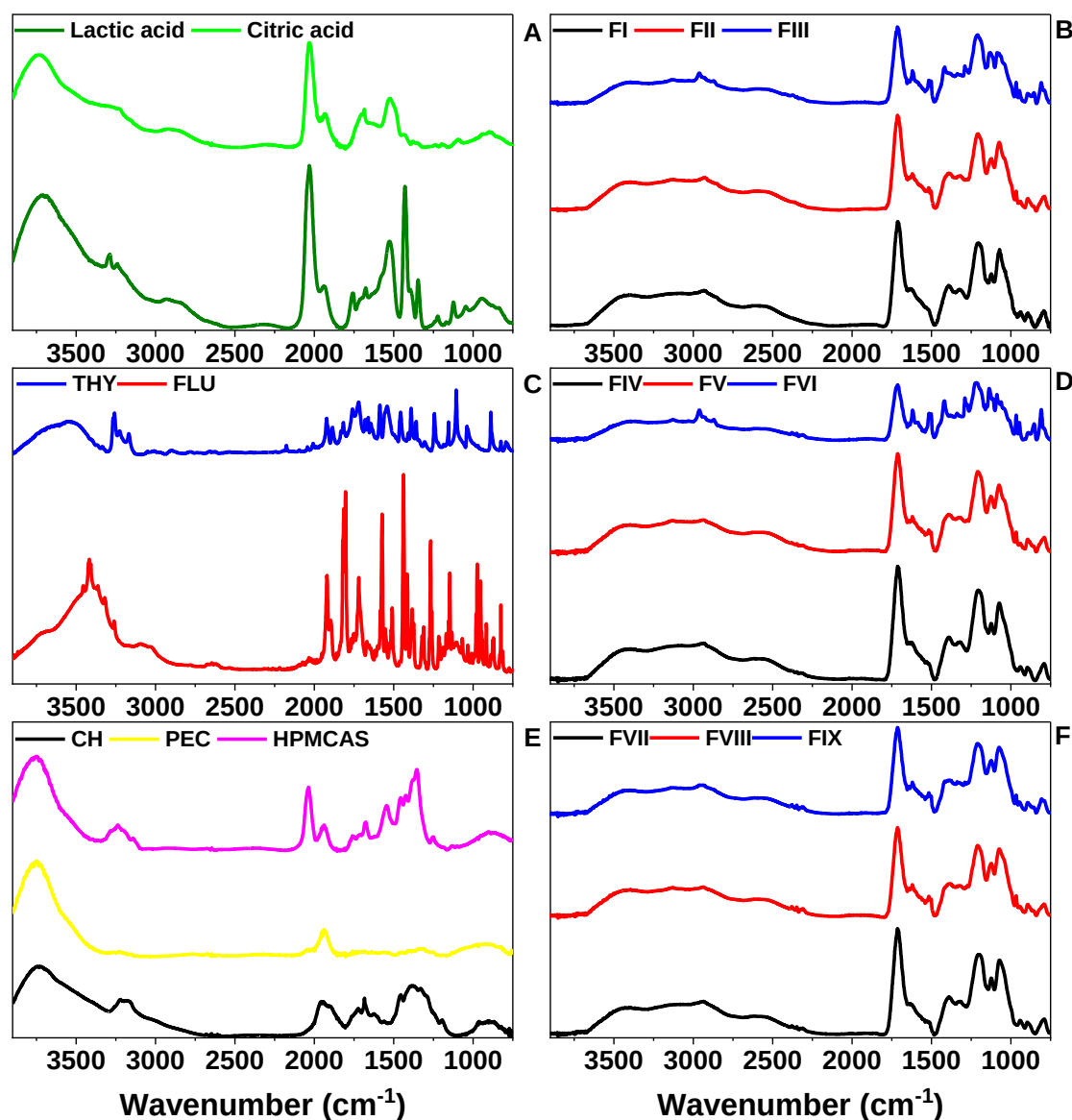


Figure 3. FT-IR spectra of raw materials (A, C and E) and polymer films (B, D and F).

3.4 X-ray diffraction

The X-ray diffraction of raw material of fluconazole is represented in Fig. S1. The X-ray diffraction patterns of the FI–FIX films are shown in Fig. 4. No recrystallization of film constituents (polymers or fluconazole) was found, which could be explained by the slow evaporation rate during the preparation of the formulations. The main crystallographic feature observed is a weak reflection at approximately 18° 2θ . This diffraction peak may correspond to the structure of citric acid and CH (Kalaimani et al., 2019; Kumar & Koh, 2012), which are in higher concentrations in the

films. This finding sustains the results from the DSC analysis (section 3.2.2), confirming that the FLU is found in an amorphous form. Similar X-ray diffraction patterns were found for polymer films with and without FLU as well as in situations where the polymer has been changed.

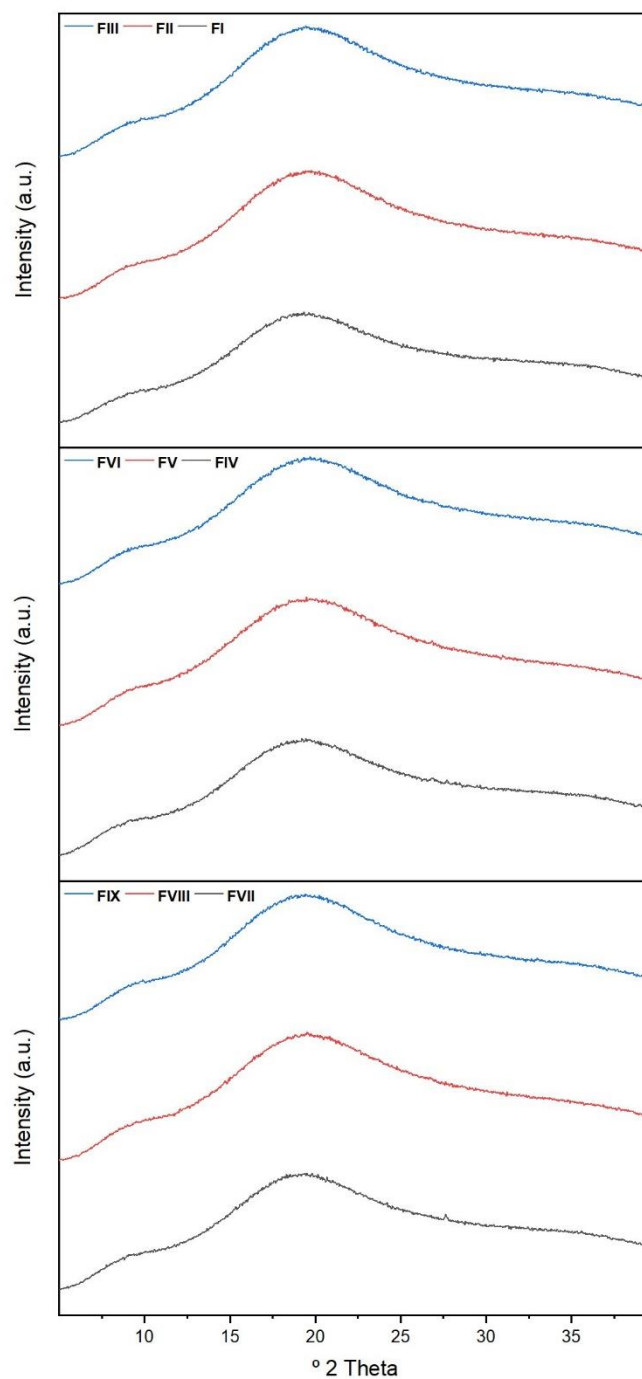


Figure 4. X-ray diffraction patterns of CH-based films (FI – FIX).

3.5 Mechanical properties

Vaginal films are designed to rapidly disperse or dissolve after contact with fluids, leading to formation of a viscous and bioadhesive gel. They should be flexible enough to facilitate the application, improving medication adherence. From the point of view of mechanical properties, they should exhibit sufficient shear and tensile strength. The type of polymer and its molecular weight affect the properties of polymer films. Plasticizers, in turn, provide more flexibility and adequate textural parameters (Notario-Pérez et al., 2020).

Stress-strain curves of CH-based films are presented in Fig 5. Young's modulus (YM), tensile strength (TS) and elongation at break (%E) were calculated from the curves, and results are presented in Table 3. CH-based films (FI – FIII) showed different values of Young's modulus, where FI showed the lower value (0.045 MPa), followed of FIII (21.12 MPa) and FII (27.799 MPa). These results demonstrate higher modulus of elasticity in FII and FIII (ability to deform and return to its original shape). FI also showed the lower TS value (0.215 MPa) whereas FII and FIII had a TS of 1.505 and 1.656 MPa, respectively. No differences into PEC/CH (FIV – FVI) and HPMCAS/CH (FVII – FIX)-based film group was observed for YM and TS. For CH-based formulations with biopolymer blends, CH interaction sites are no longer available as they are interacting with the associated polymer. Thus, few changes in mechanical properties were observed when FLU and THY were added.

The elongation percentage at the point of breaking for FI was 356% whereas FII and FIII presented percentages of 83 and 53%, respectively. The addition of FLU and THY decreases the plasticity of CH-based films, making the chains more rigid or less flexible when the force is applied. In PEC/CH-based films, only FIV was statistically different from FVI in elongation percentages (159 versus 100%). Formulations with HPMCAS had the highest %E values. FVIII had lower %E than FVII and FIX. Electrostatic interactions between pectin and chitosan in film formulations result in less plasticity than films prepared with only CH (FI) and CH/HPMCAS blend (FVII). The major interaction in HPMCAS is due to the presence of the succinate group and hydrogen bond formation. The greater free molecular volume in this molecule contributes to succinate chain stretching and sp³ carbon free rotation, resulting in a greater plasticity (Enumo et al., 2020).

FLU and THY decreased the plasticity of CH and PEC/CH-based films. Nguyen et al. (2022) have found that carvacrol (thymol isomer) and other polyphenols are able to decrease the %E of CH and pectin films (Nguyen et al., 2022). For HPMCAS/CH films with THY (FIX), competitive hydrogen interactions between thymol and succinate groups would be found, increasing the plasticity of polymer films. THY seems to fit more properly between the chains of CH and HPMCAS without significant effects in the other samples.

Several other literature studies that considered the elastic and plastic properties of CH-based films found much lower %E values (Bigi et al., 2021; Homez-Jara et al., 2018; Sanap et al., 2022; Talón et al., 2017). In these studies, acetic acid was considered for CH solubilization, which would reduce the polymer plasticity and thus the film formulations showed to be more rigid. Interestingly, the citric acid and lactic acid showed an opposite effect, an important contribution of this study given the wide application of chitosan films.

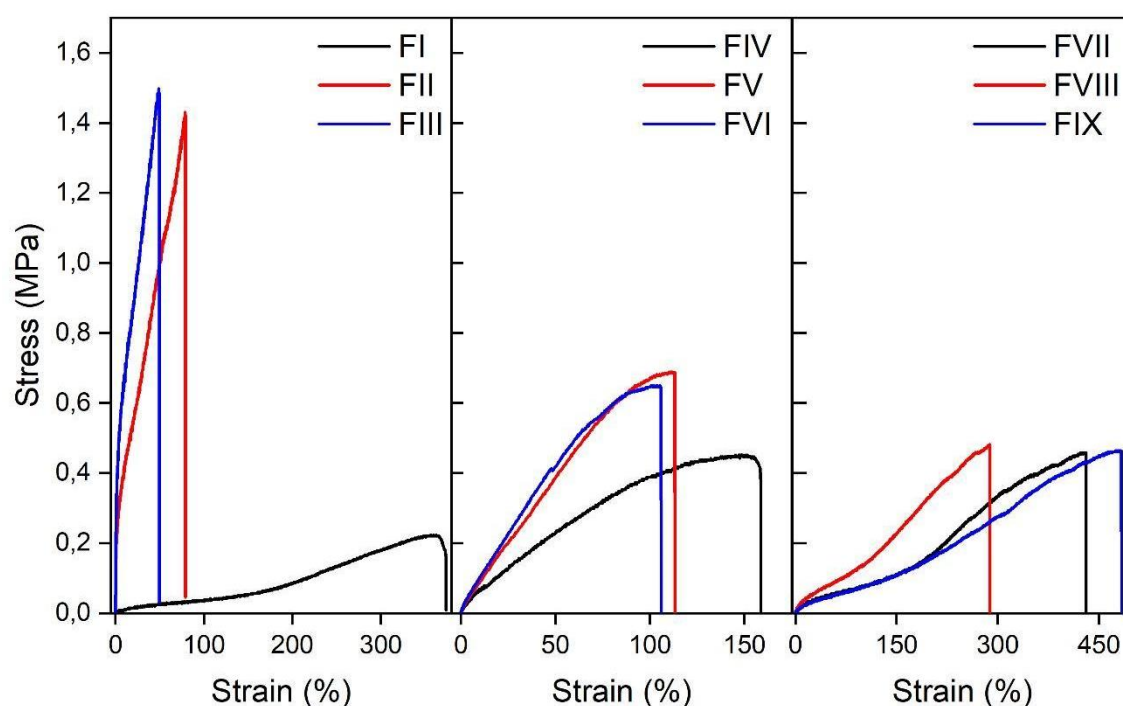


Figure 5. Stress-strain curves of chitosan films (FI - FIII), chitosan/pectin films (FIV-FVI) and chitosan/HPMCAS films (FVII-FIX).

Table 3. Tensile data of developed films ($n=5$).

Sample	Young's modulus (MPa)	Tensile strength (MPa)	Elongation at break (%)
FI	$0.045 \pm 0.005_{(a)}$	$0.215 \pm 0.006_{(a)}$	$356.106 \pm 10.276_{(a)}$
FII	$27.799 \pm 2.435_{(b)}$	$1.505 \pm 0.287_{(b)}$	$83.766 \pm 8.850_{(b)}$
FIII	$21.12 \pm 6.989_{(b)}$	$1.656 \pm 0.165_{(b)}$	$53.9 \pm 3.164_{(b)}$
FIV	$0.655 \pm 0.051_{(b)}$	$0.459 \pm 0.011_{(b)}$	$159.22 \pm 19.017_{(a)}$
FV	$1.018 \pm 0.032_{(b)}$	$0.702 \pm 0.038_{(b)}$	$119.453 \pm 4.830_{(ab)}$
FVI	$1.207 \pm 0.128_{(b)}$	$0.673 \pm 0.027_{(b)}$	$100.16 \pm 4.075_{(b)}$
FVII	$0.095 \pm 0.004_{(b)}$	$0.479 \pm 0.017_{(b)}$	$432.786 \pm 1.712_{(a)}$
FVIII	$0.191 \pm 0.029_{(b)}$	$0.475 \pm 0.030_{(b)}$	$284.033 \pm 31.242_{(b)}$
FIX	$0.085 \pm 0.003_{(b)}$	$0.460 \pm 0.049_{(b)}$	$468.053 \pm 14.686_{(a)}$

Data were expressed as mean $\pm$ SD. One-way ANOVA followed by Tukey's test ($p < 0.05$) was used to compare the same polymer composition for each tested parameter. Different letters indicate significant difference among formulations.

3.6 Wettability

The surface wettability of the polymer films was analyzed by the contact angle measurements in a goniometry (Table 4). Lower contact angle values ($<90^\circ$) indicate a tendency of water to spread and adhere to the surface, which indicate more hydrophilic films. Higher contact angle values ($>90^\circ$), in turn, indicate the surface's tendency to repel water and thus the solid material can be considered more hydrophobic.

Although statistical difference has been observed between CH-based film (FI) and other samples (FII—FIX), all films showed to have a higher wetting tendency. Films containing only CH showed greater hydrophobicity than composite films (without FLU and THY). Rotta et al. (2009) found similar findings for HPMC/CH films. The higher proportion of HPMC in CH films, more hydrophilic formulations were obtained. An opposite effect was found for increased proportions of CH. Fu et al. (2022) also found that the solubility of PEC/CH bilayer films in water was significantly reduced compared with PEC monolayer films, which demonstrates that chitosan decreases the hydrophilicity of these formulations. The addition of FLU and THY also increased the hydrophilicity of CH films, which would be associate with the reduction of interactions inter-polymers.

Table 4. Contact angle of developed films ($n=3$).

Sample	Contact angle (θ)	Sample	Contact angle (θ)	Sample	Contact angle (θ)
FI	$83.29 \pm 1.24_{(a)}$	FIV	$68.28 \pm 3.89_{(b)}$	FVII	$66.51 \pm 0.41_{(b)}$
FII	$60.41 \pm 5.14_{(b)}$	FV	$70.05 \pm 3.12_{(b)}$	FVIII	$65.12 \pm 1.20_{(b)}$
FIH	$62.49 \pm 1.49_{(b)}$	FVI	$69.36 \pm 3.89_{(b)}$	FIX	$72.34 \pm 0.79_{(b)}$

Data were expressed as mean $\pm$ SD. One-way ANOVA followed by Tukey's test ($p < 0.05$) was used to compare each film with all the others. Different letters indicate significant difference among formulations.

3.7 Swelling behavior

Drug release from matrices containing expandable polymers is essential for an efficient vaginal administration. Polymer swelling causes relaxation of the polymer chains, enhancing their penetration through the mucous membrane during the adhesion process (Bartkowiak et al., 2018). CH-based films are characterized by a rapid disintegration with the formation of a gel. In this context, swelling behavior of developed CH-based films were evaluated in different times after contact with simulated vaginal fluid in pH 4.2 at 37 °C (Fig. 6).

CH/PEC-based films (FIV, FV and FVI) showed a lower swelling index compared to CH-based films, indicating lower SVF absorption capacity after 60 min. These data corroborate with findings of Fu et al. (2022). Although PEC and CH matrices have strong hydrophilic properties, PEC has a lower capacity to expand its volume and adsorb water.

On the other hand, the association of HPMCAS with CH increased the swelling capacity of the films compared to films composed only of CH. Polymer blends increase free volume and thus the flexibility of the polymer chains. Furthermore, the HPMCAS succinate groups have free rotation, which can modify the film properties and provide greater water adsorption capacity (Sanap et al., 2022). The association of HPMCAS with CH provides ideal characteristics for application in the vaginal mucosa since the crosslinking of HPMCAS with CH could provide a sustained release of drugs.

The swelling behavior between films of the same polymeric composition correlates with the wettability data. In the CH and CH/HPMCAS films, the lowest contact angle values (more hydrophilic) showed greater swelling capacity. For the

CH/PEC films, contact angle values close each other were obtained, which is also found in the swelling profile analysis.

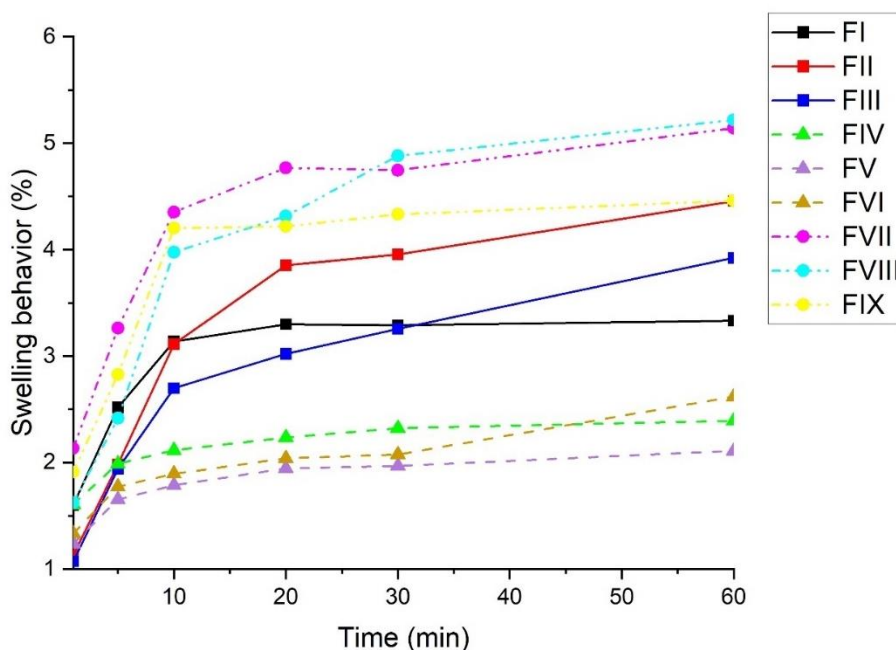


Figure 6. Swelling behavior of developed films from FI to FIX ($n=3$).

3.8 Scanning electron microscopy (SEM)

SEM images of the surface and cross-sectional fracture of the developed films were obtained for a morphological analysis (Fig. 7). All polymer matrices showed a homogeneous and smooth surface, except for the FIII. This formulation presented a deposition of a constituent of the film, which is probably thymol as it is not found in FI and FII. An improved distribution of this agent would be observed in the matrices when the CH is crosslinked to PEC and HPMCAS. FLU crystals were not observed on films, corroborating the results obtained from the X-ray diffraction (section 3.4).

In cross-sectional fracture, HPMCAS films (FVII, FVIII and FIX) showed a more porous polymer matrix, which corroborates the results of plasticity described in the section 3.5. All polymer films showed a more uniform morphology than other developed films composed of the same biopolymers reported in literature (Calvo et al., 2019; Mishra et al., 2017), which could be associated with the CH solubilization step in different acids (e.g., acetic acid and lactic acid only).

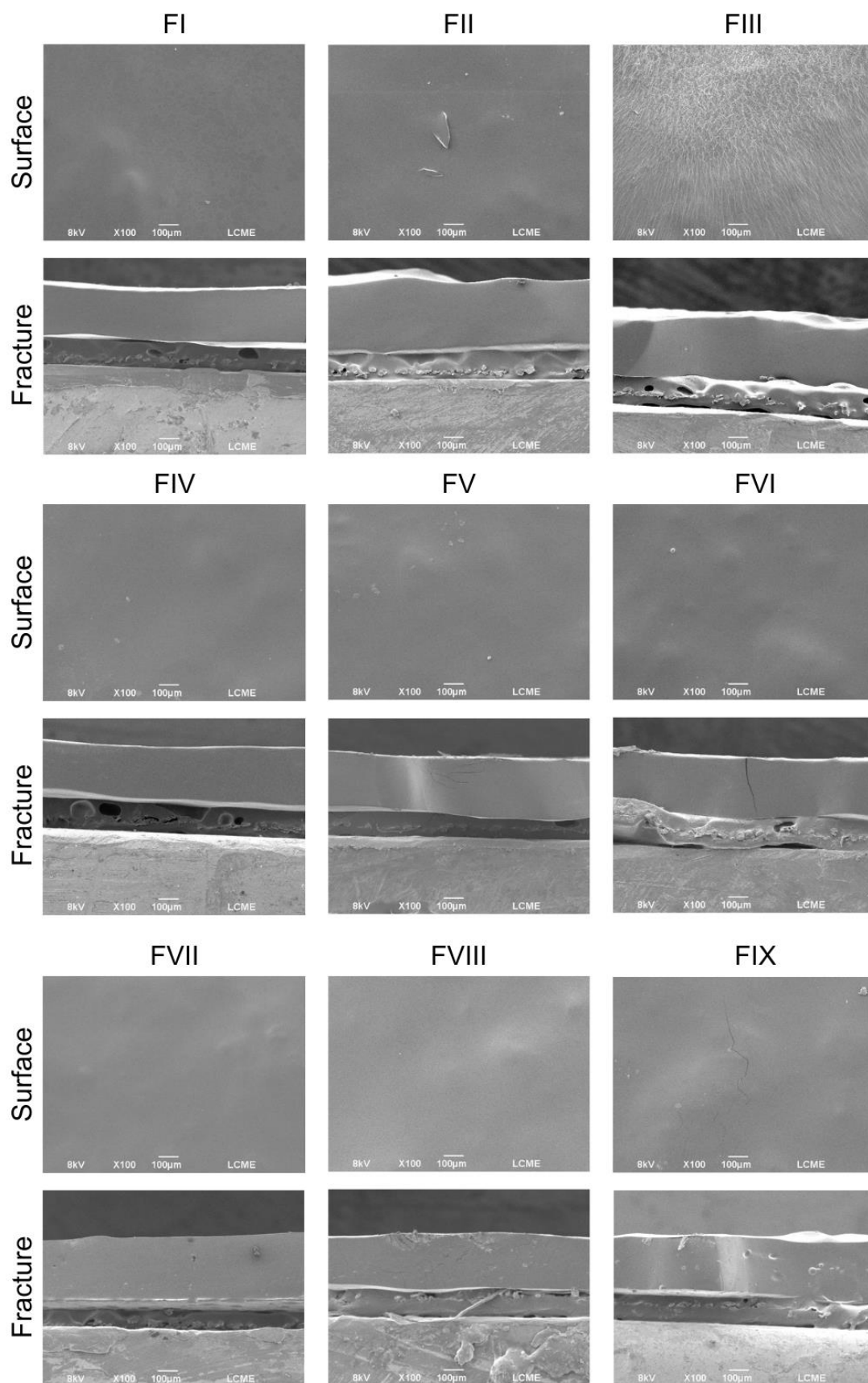


Figure 7. SEM images of surface and cross-sections of films.

3.9 Ex vivo permeability study

FII, FIII, FVI and FIX were selected for these assays aiming to evaluate the effect of both thymol and polymer matrix on the vaginal permeability of FLU (Fig. 8; Table 5). A FLU solution (1.1 mg mL^{-1} in water) was used as control.

After treatment with the control, the permeability flux of FLU through the vaginal mucosa was $12.29 \pm 0.73 \text{ } \mu\text{g/cm}^2\cdot\text{h}$, presenting a lag time of $1.77 \pm 0.21 \text{ h}$ and a permeability coefficient of $5.59 \times 10^{-3} \pm 0.33 \times 10^{-3} \text{ cm/h}$. All tested films provided an increased permeability of FLU through vaginal mucosa. The flux rate of FLU increased in approximately 3.9-, 5.8-, 6.6- and 5.3-fold after treatment with FII, FIII, FVI and FIX, respectively.

A shorter lag time was found for all developed polymer films, which were statistically significant differences when compared to control ($p < 0.05$). These results indicate a shorter period for the tissue to become saturated with the drug, suggesting a faster onset therapeutic activity when films are applied. All CH-based films also showed higher permeability coefficient values than the control ($p < 0.05$). The presence of CH in polymer films could explain these findings. In addition to enhanced interaction with the tissue, CH would play a role in the translocation of tight junction proteins from the membrane to the cytoskeleton (Smith et al., 2004).

FIII provided greater flux and permeability coefficient for the FLU than the FII, suggesting that THY plays a role as absorption enhancer. THY has already been shown to improve mucosal and topical drug permeability in previous studies (Chantasart et al., 2009; Amaral et al., 2020). Amaral et al. (2021), for example, demonstrated that the presence of thymol increases the erosion of polymer matrices and also prevents drug crystallization in solution during mucosal transport studies.

FVI provided the highest transport rate for the FLU, which can be attributed to a rapid film disintegration and its low swelling capacity. Although film-forming polymers (CH and PEC) interact electrostatically, a rapid gel formation is observed after contact with the vaginal fluid as already reported (Mishra et al., 2017).

The amount of FLU retained in the vaginal mucosa was also evaluated (Fig. S2). FVI provided the highest drug retention in the tissue ($110.58 \pm 13.70 \text{ } \mu\text{g/cm}^2$), followed by FII ($54.40 \pm 7.23 \text{ } \mu\text{g/cm}^2$), FIII ($38.54 \pm 9.56 \text{ } \mu\text{g/cm}^2$), FIX ($33.13 \pm 3.51 \text{ } \mu\text{g/cm}^2$) and control solution ($27.93 \pm 1.36 \text{ } \mu\text{g/cm}^2$). All polymer films enhanced the retention of FLU in the mucosa and thus an improved anti-*Candida* activity would be expected.

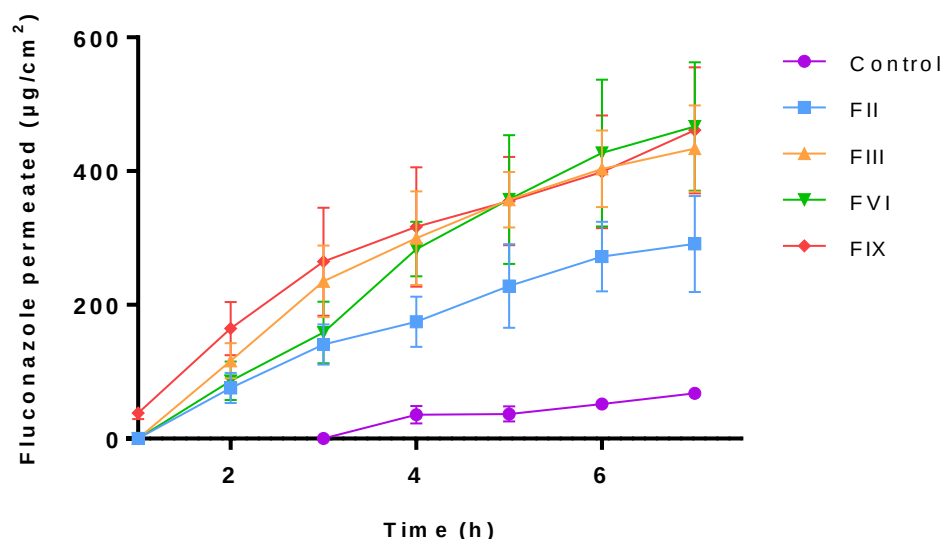


Figure 8. Permeability profile of fluconazole from polymeric films through the porcine vaginal mucosa. Data are presented as mean $\pm$ standard deviation ($n = 5$).

Table 5. Permeability parameters of fluconazole from the control solution and film formulations.

	J_{ss} ($\mu\text{g}/\text{cm}^2\cdot\text{h}$)	T_L (h)	P ($\text{cm}/\text{h} \times 10^{-3}$)
Control	12.29 ± 0.73	1.77 ± 0.21	5.59 ± 0.33
FII	$48.34 \pm 11.18^*$	$0.50 \pm 0.14^*$	$21.97 \pm 5.08^*$
FIII	$71.34 \pm 10.94^*$	$0.46 \pm 0.24^*$	$32.43 \pm 4.97^*$
FVI	$81.43 \pm 17.98^*$	$0.87 \pm 0.08^*$	$37.01 \pm 8.17^*$
FIX	$65.31 \pm 15.00^*$	$0.62 \pm 0.25^*$	$29.69 \pm 6.82^*$

Data were plotted as mean $\pm$ standard deviation. Treatments were compared by one-way ANOVA with Tukey's post-test ($n=5$) (each parameter was analyzed separately). Values of $p < 0.05$ were considered statistically significant. *indicate statistically significant differences compared to control. J_{ss} = steady state flux; T_L = lag time; P = permeability coefficient.

3.10 ATR-FTIR analysis of vaginal mucosa

The same film formulations selected for permeability studies (FII, FIII, FVI and FIX) were placed in contact with the vaginal mucosa to investigate the effect of them in disorder degree of protein and lipid domains. PBS-treated tissue was used as control

(blank). After 7 h of treatment, the tissue samples were analyzed by ATR-FTIR and the collected spectra are represented in Fig. 9.

The characteristic bands related to proteins can be observed in the control spectrum (blank) at $1,639\text{ cm}^{-1}$ (vibration of amide I), $1,552\text{ cm}^{-1}$ (vibration of amide II) and $3,285\text{ cm}^{-1}$ (amides of proteins). The effect of THY on FIII, FVI and FIX seems to reduce the intensity of amide I and II bands. Moreover, THY shifted the amide I wavenumber to $1,624\text{ cm}^{-1}$, which corresponding to a protein secondary structure of β -sheet. For the control tissue, this band was associated with a random coil structure (Amaral et al., 2020). The amides of proteins shifted from $3,285\text{ cm}^{-1}$ (untreated tissue and FII) to $3,272\text{ cm}^{-1}$ (FIII, FVI and FIX), suggesting a lower degree of order in the protein domains of these samples. These results corroborate the results of permeability studies. The formulations with THY showed a higher FLU transport rate and protein disorder, sustaining the hypothesis of its action as an absorption enhancer agent.

The bands related to lipids were not demonstrated to be affected by the presence of thymol. In all samples, the symmetric CH_2 stretching vibrations are showed at $2,850\text{ cm}^{-1}$, which indicates a dominant orthorhombic arrangement of the vaginal mucosa (Schwarz et al., 2013). Asymmetric CH_2 stretching vibrations, which appear at $2,923\text{ cm}^{-1}$, also does not seem to be affected by the treatment of polymer films. CH_2 scissoring mode is represented by only single peaks in mucous membranes, indicating a largely hexagonal structure (Caussin et al., 2008). In untreated mucosa (blank), CH_2 scissoring vibrations, which are showed at $1,456\text{ cm}^{-1}$, seems to have a slight reduction on intensity after treatment with FII, FIII, FVI and FIX. This finding suggests a certain lipid disorder caused by these samples.

The analysis of protein and lipid bands of the spectra also reinforces the results obtained in the permeability studies since the transport rate of FLU through the films was higher than that of control solution. Moreover, as already mentioned, CH in polymeric films can be able to translocate tight junction proteins (Smith et al., 2004).

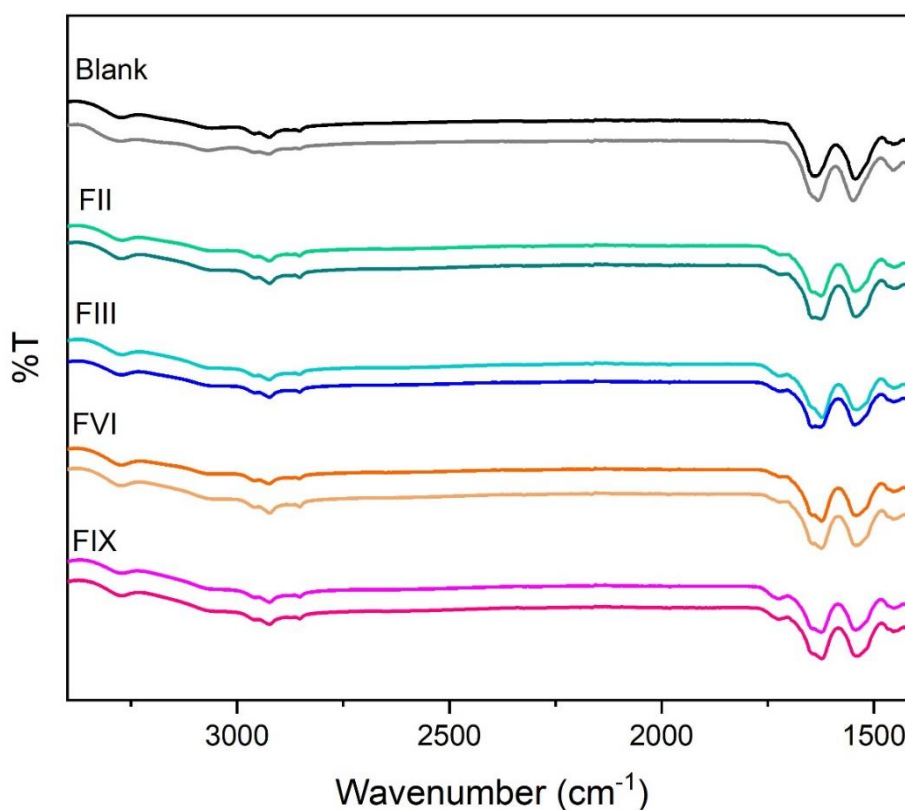


Figure 9. ATR-FTIR spectra ($n=2$) of porcine vaginal mucosa after treatment with PBS (blank/control) and FII, FIII, FVI and FIX.

3.11 *In vitro* antifungal activity

The first step of this study included the evaluation of the minimum inhibitory concentration (MIC) of CH films prepared only with FLU or THY by using the disk-diffusion method. FI (formulation without active compounds) was used as a control and did not show any halo of inhibition against the tested strain. For the FLU, MIC values of 256, 4, 256 and 512 $\mu\text{g}/\text{disk}$ against *C. albicans*, *C. parapsilosis*, *C. krusei* and *C. glabrata* strains were found. THY, in turn, showed a MIC value of 2,048 $\mu\text{g}/\text{disk}$ against *C. glabrata*. On the other hand, it did not show to be active against *C. albicans*, *C. parapsilosis* and *C. krusei* strains in the tested concentrations (4 – 2,048 $\mu\text{g}/\text{disk}$). This finding is interesting from a clinical point of view given that many episodes of microbial resistance have been associated to *C. glabrata* (Sobel, 2023).

After the determination of MIC against the *C. glabrata* for CH films with each active compound (512 and 2,048 $\mu\text{g}/\text{disk}$ for FLU and THY, respectively), formulations containing both FLU and THY, at these same concentrations, were developed aiming to evaluate if an additive, antagonistic or synergistic effect would be found. Furthermore, polymer films with subinhibitory concentrations of these active compounds (256 and

1,024 $\mu\text{g}/\text{disk}$) were also considered to evaluate the possibility of dose reduction so that the same antifungal efficacy would be achieved. The diameter values of the inhibition zone are reported in Fig. 10. Similar areas were obtained ($p>0.05$).

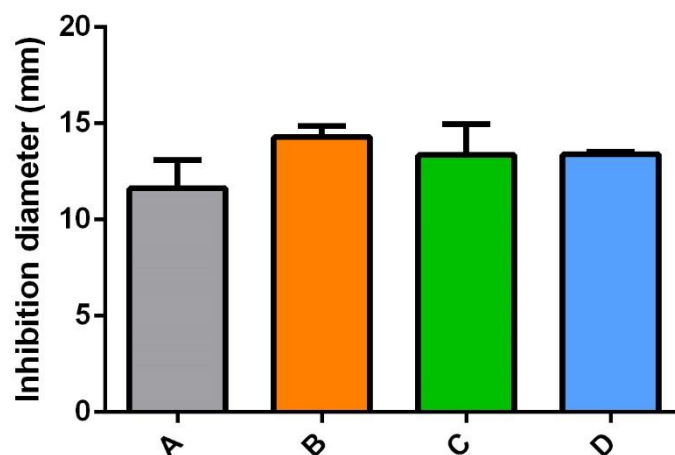


Figure 10. Anti-*Candida* activity of chitosan-based films loaded with fluconazole (FLU) and/or thymol (THY) against *C. glabrata* strain ($n=3$); **(A)** CH-film with FLU (512 $\mu\text{g}/\text{disk}$); **(B)** CH-film with THY (2,048 $\mu\text{g}/\text{disk}$); **(C)** CH-film with FLU and THY (512 and 2,048 $\mu\text{g}/\text{disk}$, respectively) and **(D)** CH-film with FLU and THY (256 and 1,024 $\mu\text{g}/\text{disk}$, respectively).

CH films containing only FLU at 256 $\mu\text{g}/\text{disk}$ or containing only THY at 1,024 $\mu\text{g}/\text{disk}$ did not show any inhibition against *C. glabrata* (data not shown). On the other hand, the combination of FLU and THY at subinhibitory concentrations resulted in an inhibition halo of 13.4 mm. Once this association of active compounds at subinhibitory concentration presented similar antifungal efficacy to formulations prepared with the combination of these compounds in their respective MIC values, a probable synergistic or additive action would be found.

Although the *C. glabrata* are more prone to develop resistance to FLU than other *Candida* spp (Pfaller et al., 2014), studies have demonstrated that the association of FLU with natural compounds can reduce its MIC against this *Candida* strain (Machado et al., 2016; Machado et al, 2021; Pippi et al., 2015). When two compounds are able to reach microbial cells by two or more different targets/mechanisms, an improved efficacy can be found. FLU is known for acting on the biosynthesis of ergosterol whereas multiple mechanisms seem to be involved in THY action, which include increased membrane permeability, inhibition of protein and DNA synthesis as well as mitochondrial membrane disintegration (Karpiński, 2020). Taken together, these

results suggest that polymer films containing this active combination can be promising for the treatment of VVC, particularly in resistant infections caused by *C. glabrata*.

4 FINAL CONSIDERATIONS

Biopolymer films were successfully obtained through the solvent evaporation method. The addition of PEC and HPMCAS to chitosan films did not affect their thermal properties and materials in an amorphous state were obtained. This last characteristic would contribute to a rapid solubilization and disintegration of these thin films in vaginal fluid. Interestingly, the use of citric acid and lactic acid as solubilizing agents of chitosan, which had not yet been tested, resulted in more flexible films than CH films prepared with only acetic acid. CH/HPMCAS films showed greater plasticity and flexibility than systems prepared with PEC or only chitosan, which explain the higher swelling index in simulated vaginal fluid. All polymer films provided high permeability rates of FLU through the vaginal mucosa, which is probably associated with the action of chitosan in loosening adherent junctions found among epithelial cells. THY showed to be an effective chemical absorption enhancer, increasing the distribution of FLU in tissues. The incorporation of thymol in CH films was also advantageous to improve the antifungal activity of FLU. In fact, this combination was active against *C. glabrata*, a resistant strain of the *Candida* genus that causes recurrent VVC.

Taken together, these results demonstrated that polymer films are a promising alternative for the treatment of resistant vulvovaginal candidiasis, encouraging clinical trials. CH/HPMCAS films should be prioritized due to improved mechanical properties and higher swelling ratio, which would make application easier and provide a more sustained drug release.

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Declaration of Competing Interest

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.

Authors contributions

Júlia Conte: Conceptualization; Investigation; Methodology; Writing - Original Draft; Rodrigo Henrique Saatkamp: Conceptualization; Investigation; Methodology; Writing - Original Draft; Mariele Paludetto Sanches: Investigation; Methodology; Débora Fretes Argenta: Investigation; Gabriella da Rosa Monte Machado: Investigation; Methodology; Iara Fabrícia Kretzer: Investigation; Methodology; Alexandre Luis Parize: Methodology; Review & Editing; Thiago Caon: Conceptualization; Methodology; Writing - Review & Editing; Supervision.

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5.2 MATERIAL SUPPLEMENTAR

**BIOPOLYMER COMPOSITE FILMS BASED ON CHITOSAN FOR
RESISTANT VAGINAL CANDIDIASIS**

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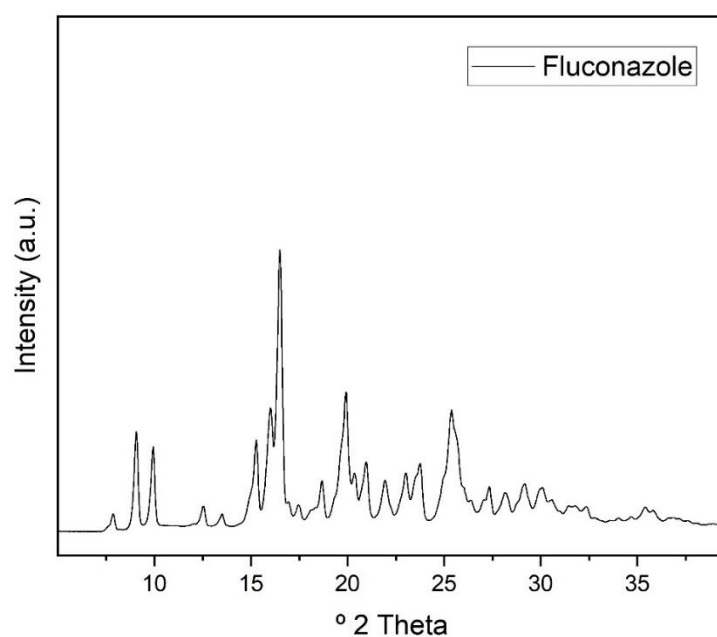
Supplementary Table S1. Thermal events in TG analysis of pure constituents and polymer films.

Sample	Thermal event						Residue (%)
	1°		2°		3°		
	Temperature (°C)	Weight loss (%)	Temperature (°C)	Weight loss (%)	Temperature (°C)	Weight loss (%)	
Pectin	20 to 175	15.20	190 to 440	51.32	448 to 600	13.10	20.38
HPMCAS	20 to 94	1.02	190 to 425	88.12	537 to 773	8.43	2.43
Chitosan	40 to 139	1.82	250 to 425	51.00	500 to 800	30.00	17.18
Fluconazole	70 to 95	1.85	200 to 330	97.48	-	-	0.67
Thymol	60 to 188	99.50	-	-	-	-	0.50
Citric acid	61 to 95	2.44	141 to 167	5.04	186 to 306	92.12	0.40
Latic acid	31 to 90	16.29	105 to 265	82.02	-	-	1.69
FI	40 to 310	55.20	310 to 550	27.92	-	-	16.88
FII	40 to 310	55.60	310 to 550	27.43	-	-	16.97
FIII	40 to 310	56.50	320 to 550	26.56	-	-	16.94
FIV	40 to 310	56.60	310 to 600	28.53	-	-	14.87
FV	40 to 310	55.27	310 to 600	25.30	-	-	19.43
FVI	40 to 310	55.41	310 to 600	27.25	-	-	17.34
FVII	50 to 300	57.00	300 to 600	21.86	-	-	21.14
FVIII	50 to 316	54.00	321 to 600	28.90	-	-	17.10
FIX	50 to 309	56.82	316 to 600	26.82	-	-	16.36

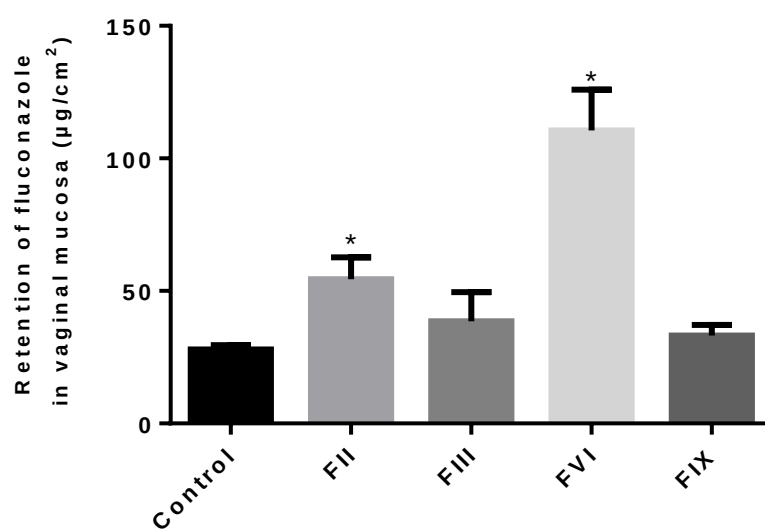
Supplementary Table S2. Thermal characteristics of the film and pure constituents obtained from DSC analyses.

Sample	Thermal event								
	T _{m1} (°C)	ΔH ₁ (J g ⁻¹)	T _{m2} (°C)	ΔH ₂ (J g ⁻¹)	T _{m3} (°C)	ΔH ₃ (J g ⁻¹)	T _c	Tg 1	Tg2
Pectin	106	463.2	-	-	-	-	240.3	-	-
HPMCAS	97.36	99.93	288	97.4	-	-	-	58	-
Chitosan	-	-	-	-	-	-	-	70	200
Fluconazole	77	52.74	101	3.5	141	120	-	-	-
Thymol	52.3	128.5	-	-	-	-	-	-	-
Citric acid	60.95	117.7	225	642.4	-	-	-	-	-
Latic acid	97.8	340.4	211.4	268.4	-	-	-	-	-
FI	171	596.6	-	-	-	-	-	-	-
FII	162	104	172	296.9	-	-	-	-	-
FIII	167	526	-	-	-	-	-	-	-
FIV	176	489	-	-	-	-	-	-	-
FV	182	440.1	-	-	-	-	-	-	-
FVI	141.6	586.9	-	-	-	-	-	-	-
FVII	151	675.4	-	-	-	-	-	-	-
FVIII	173	504.9	-	-	-	-	-	-	-
FIX	155	146.6	198	300.6	-	-	-	-	-

T_m – Melting temperature; T_c – Crystallization temperature; T_g – Glass transition temperature.



Supplementary Figure S1. X-ray diffraction pattern of crystalline fluconazole.



Supplementary Figure S2. Fluconazole retained in porcine vaginal mucosa after 7h. Values are represented as mean $\pm$ standard deviation (n = 5). *indicate statistically significant differences compared to control.

6 DISCUSSÃO GERAL

A administração oral de antifúngicos azólicos, durante o tratamento da candidíase vaginal, pode levar a interações medicamentosas e inúmeros efeitos adversos (NSENGA & BONGOMIN, 2022). Ainda, o uso irracional e indiscriminado dos azóis pode acentuar problemas de resistência fúngica, requerendo doses maiores ou então terapias combinadas (GILHOTRA *et al.*, 2014). Parte destes problemas podem ser resolvidos com a troca da via de administração. Neste caso, a seleção da rota vaginal parece ser uma estratégia promissora tendo em vista a proximidade do sítio de ação. Com isto, pacientes não seriam expostas a riscos sistêmicos indesejáveis. A rota vaginal evita o metabolismo de primeira passagem, o que permite uma redução da dose administrada e então de efeitos adversos associados. Além disto, um maior controle da liberação de ativos pode ser alcançado (MACHADO *et al.*, 2015). Com relação a resistência fúngica, a associação de ativos pode ser uma alternativa promissora, o que foi explorado neste novo estudo.

Cremes, géis e pomadas vaginais tem sido as formas farmacêuticas mais comuns do mercado devido a simplicidade na produção. Por outro lado, extravasamento do canal vaginal, alta frequência de administração, baixa adesão ao tratamento, falta de uniformidade de conteúdo, baixa biodisponibilidade e estabilidade são problemas que são frequentemente observados com formulações semissólidas (JOHAL *et al.*, 2016). Por outro lado, formas de dosagem sólidas podem contornar estes problemas, o que foi explorado neste trabalho. Assim, a primeira etapa desta dissertação consistiu em realizar uma revisão teórica (capítulo II) de formulações sólidas “inovadoras” que tem sido empregadas no tratamento da candidíase vaginal. Anéis vaginais, filmes, esponjas e nanofibras poliméricas foram as formas farmacêuticas priorizadas.

Anéis vaginais são sistemas que permitem alcançar diferentes perfis de liberação e por longos períodos. Esta característica é essencial em casos de resistência microbiana. Por outro lado, fármacos potentes ou de baixa dosagem devem ser priorizados. O processo de produção é complexo, com menor variedade de polímeros que podem ser considerados devido a necessidade de propriedades mecânicas específicas, além de limitações relativas as formas e tamanhos dos anéis. Os anéis devem ser flexíveis para permitir uma aplicação eficiente e não devem ser duros a ponto de lesionar a mucosa.

As esponjas poliméricas, por sua vez, são caracterizadas por alta porosidade ou área de superfície, o que garante alta interação polímero-mucosa (mucoadesão) e alta taxa de incorporação de fármacos na sua estrutura. São particularmente úteis nas situações em que se almeja uma ação mais rápida. Como a estrutura é porosa e, desta forma, mais frágil, rápida desintegração é comumente encontrada. Por outro lado, a sua maior espessura pode causar certo desconforto à paciente e são sistemas menos estáveis.

As nanofibras poliméricas, que são obtidas pela deposição de fibras eletrofiadas, também proporcionam alta interação polímero-mucosa e permitem a incorporação de alto teor de fármaco. Por outro lado, o processo de produção envolve várias etapas e exige um rigoroso controle dos parâmetros de processo a fim de preservar as características das nanofibras. A transposição de escala é sempre muito desafiante para estes sistemas.

Filmes poliméricos tem sido amplamente explorados devido a versatilidade de técnicas e materiais que podem ser empregados durante a etapa de preparo. Ainda, podem assumir várias formas, tamanhos, espessura e são mais facilmente escalonáveis. Quando polímeros bioadesivos são selecionados, maior tempo de residência dos filmes no sítio de aplicação é encontrado. As dimensões do filme polimérico têm relação com a potência do fármaco, quantidade total que pode ser incorporada e efetivamente liberada. Todos estes aspectos motivaram a seleção deste tipo de formulação para a etapa experimental dessa dissertação (capítulo III), que envolveu o preparo e caracterização de filmes biopoliméricos de quitosana. Polímeros como a pectina e HPMCAS foram também combinados com a quitosana. Fluconazol e timol foram selecionados como ativos.

O timol, composto majoritário do óleo essencial de tomilho, além de apresentar ação promotora de permeação em tecidos mucosais e pele (CHANTASART *et al.*, 2009; AMARAL *et al.*, 2020), já demonstrou ação sinérgica com o fluconazol frente a cepas de *Candida* (JAFRI & AHMAD 2020).

O método de evaporação de solvente se mostrou adequado para o preparo dos filmes dada a completa solubilização dos componentes na solução filmogênica, sem que fosse observada recristalização do fluconazol. Isto pode ter relação com a lenta taxa de secagem. Durante o preparo dos filmes, diferentes tipos de ácidos foram testados para a solubilização da quitosana. A literatura traz, na maior parte dos estudos, a solubilização com ácido acético, o que implica em filmes quebradiços. De forma inovadora, diferentes ácidos foram aqui testados. Ácidos que resultaram em filmes quebradiços,

com cristais de fármacos e baixa adesividade foram descartados. A combinação de ácido cítrico (3%) e ácido láctico (0,25%), por sua vez, se mostrou promissora como filmes com características macroscópicas de maleabilidade e adesividade foram obtidos (aspectos-chaves para a aplicação almejada).

Nove filmes poliméricos foram desenvolvidos incluindo diferentes composições a fim de avaliar como a associação polimérica e a adição de fluconazol e timol afetam suas características. Todos as formulações apresentaram uma espessura compatível com a aplicação vaginal (~0,32 mm). Os filmes de quitosana/pectina e quitosana/HPMCAS mostraram uma espessura ainda menor que filmes compostos apenas por quitosana. A reticulação, que envolve interações eletrostáticas da quitosana (carga positiva) com a pectina ou HPMCAS (carga negativa) ou ainda a formação de ligações de hidrogênio, explica estes achados. Com estas interações, o espaçamento entre as cadeias poliméricas é reduzido e, conseqüentemente, a espessura dos filmes (SOPYAN *et al.*, 2022).

Análises de FT-IR demonstraram que a quitosana e os biopolímeros selecionados foram miscíveis e compatíveis entre si. Bandas características do fluconazol e do timol foram identificadas nos espectros dos filmes poliméricos em que estes compostos ativos foram adicionados, sugerindo uma efetiva incorporação. Ainda, observou-se uma redução na intensidade das bandas de IR nos filmes que continham timol. Esta observação também foi descrita por Talón e colaboradores (2017), onde os autores sugerem que a molécula de timol aumentaria a interação entre as cadeias poliméricas.

A adição de pectina e HPMCAS aos filmes de quitosana também não afetou suas propriedades térmicas. Por outro lado, análises de difração de raios X de pó mostraram que a cristalinidade dos polímeros e do fármaco foi reduzida após o preparo de filmes, o que explicaria a rápida solubilização e desintegração deles. Considerando que a mucosa vaginal é caracterizada por uma quantidade de fluido muito baixa (± 6 mL/dia), é crucial que os filmes sejam capazes de se desintegrar rapidamente. Imagens de microscopia eletrônica de varredura, por sua vez, mostraram que os filmes apresentam uma superfície homogênea, uniforme e não-porosa, com a ausência de cristais do fármaco (corroborando com os resultados de difração de raios X).

Análises da força de tração e porcentagem de alongamento foram realizadas a fim de avaliar as propriedades mecânicas dos filmes. Todas as formulações mostraram propriedades plásticas e elásticas mais adequadas à administração vaginal que filmes de quitosana da literatura (BIGI *et al.*, 2021; HOMEZ-JARA *et al.*, 2018; SANAP *et al.*,

2022; TALÓN *et al.*, 2017). A forma de solubilização da quitosana parece ser uma condição chave para explicar estas diferenças. Quando o ácido acético é considerado, filmes mais rígidos são obtidos. A combinação de ácido cítrico e ácido lático usada neste trabalho parece ter atuado como plastificante, melhorando as propriedades mecânicas dos filmes sem a necessidade de adicionar um agente específico para tal finalidade.

A adição de fluconazol e timol às matrizes poliméricas diminuiu a porcentagem de alongamento dos filmes preparados somente com quitosana ou com pectina/quitosana, diminuindo assim a plasticidade. Resultados similares foram encontrados por Nguyen e colaboradores (2022). Por outro lado, a adição destes agentes a filmes de quitosana/HPMCAS aumentou a plasticidade do filme, provavelmente devido a interações competitivas de hidrogênio entre o timol e o grupo succinato presente na estrutura de HPMCAS. Em resumo, os filmes de quitosana/HPMCAS mostraram maior plasticidade e flexibilidade do que os sistemas preparados com pectina ou apenas quitosana, o que explica a maior taxa de intumescimento após o contato com o fluido vaginal simulado.

Além de melhorar as propriedades mecânicas, a combinação dos biopolímeros tornou os filmes mais hidrofílicos. De fato, ensaios de molhabilidade revelaram que filmes preparados apenas com quitosana apresentaram maior ângulo de contato após a deposição de água na sua superfície, demonstrando maior hidrofobicidade em relação aos filmes com as combinações biopoliméricas. Assim, filmes de quitosana com HPMCAS ou pectina demonstrariam maior afinidade pelo fluido vaginal, explicando uma maior liberação de ativos, particularmente quando se consideram mecanismos de difusão.

A permeabilidade vaginal do fluconazol através dos filmes foi investigada em modelo de mucosa vaginal suína e células de difusão de Franz. Todas as formulações selecionadas para este ensaio proporcionaram altas taxas de permeabilidade para o fluconazol, o que pode ser resultado da ação da quitosana no afrouxamento das junções aderentes entre as células epiteliais (SMITH *et al.*, 2004), que fisiologicamente explicam as propriedades de barreira dos tecidos mucosais. O timol mostrou ser um promotor de absorção químico eficaz, aumentando a distribuição do fluconazol nos tecidos. O filme com quitosana e pectina apresentou a maior permeabilidade e retenção do fármaco no tecido, o que pode estar relacionado com a rápida desintegração do filme

e baixa capacidade de intumescimento. Ambos os eventos contribuem para a rápida liberação do fluconazol a partir das matrizes poliméricas.

Para investigar como os filmes poliméricos afetam domínios lipídicos e proteicos da mucosa vaginal, espectros de infravermelho de amostras destes tecidos tratados com os filmes foram analisados. O tempo de tratamento foi o mesmo daquele dos ensaios de permeação e tecido tratado apenas com tampão fosfato salina foi utilizado como controle. Formulações com timol promoveram maior desordem dos domínios proteicos, reforçando a hipótese da sua ação como promotor de permeação. Em comparação ao tecido tratado com tampão fosfato salina (controle), pode-se observar uma maior desordem lipídica nos tecidos tratados com os filmes poliméricos. Concluindo, alterações em domínios polares foram mais evidentes, o que pode ter relação tanto com o efeito promotor do timol (interação tipo hidrogênio com o tecido) quanto com o mecanismo de ação da quitosana (interações eletrostáticas) e da rota de transporte utilizada pelo fármaco (fluconazol tem características mais polares e, desta forma, seria transportado preferencialmente pela rota intercelular).

A incorporação do timol nos filmes de quitosana também mostrou ser vantajosa para melhorar a atividade antifúngica do fluconazol. Essa combinação foi ativa frente a *C. glabrata*, que é uma cepa resistente de grande importância clínica devido a altas taxas de prevalência. De fato, é a segunda mais prevalente entre as pacientes, ficando atrás apenas da *C. albicans* (SOBEL, 2023). Com a associação de timol e fluconazol, a dose efetiva para inibir o crescimento da *C. glabrata* foi reduzida em 50%. Cabe ressaltar que fluconazol a 256 µg/disco e timol a 1024 µg/disco (quantidades utilizadas na associação), quando testados isoladamente, não inibiram o crescimento da *C. glabrata*. Isso ressalta a importância da associação para atividade frente a este microrganismo.

Em resumo, a administração vaginal de filmes poliméricos se mostrou uma opção vantajosa para o tratamento da candidíase vaginal. Além de evitar o efeito de primeira passagem hepática, podem aumentar o tempo de residência na mucosa e alcançar o sítio de ação de forma mais direcionada. Isto permitiria reduzir a dose administrada, com impacto direto nos efeitos adversos. Os filmes apresentaram propriedades mecânicas superiores à de outros filmes de quitosana da literatura, o que traria vantagens durante a aplicação. Ainda que rápida desintegração tenha sido observada nos ensaios *in vitro*, uma desintegração lenta seria esperada *in vivo* devido as diferenças na quantidade de fluido vaginal. Isto garantiria uma liberação do ativo de

forma mais sustentada, o que é crucial em terapias antimicrobianas, particularmente nos casos de resistência microbiana. A atividade frente a *C. glabrata* também encoraja a realização de ensaios clínicos. Filmes de quitosana/HPMCAS deveriam ser priorizados neste estágio como apresentaram propriedades mecânicas mais compatíveis com esta aplicação e maior taxa de intumescimento, o que poderia resultar em uma liberação de ativo mais sustentada.

7 CONSIDERAÇÕES FINAIS

-O método de evaporação do solvente com secagem lenta se mostrou adequado para produzir filmes vaginais poliméricos compostos com fluconazol e timol.

-A associação dos ácidos cítrico e lático para a solubilização da quitosana resultou em filmes com maleabilidade e adesividade compatíveis à aplicação vaginal.

-Filmes com espessura reduzida e uniformes foram obtidos. A reticulação entre os biopolímeros levou a uma redução da espessura dos filmes.

-Através de análise de FT-IR, DSC e TGA não indicaram incompatibilidades entre os componentes selecionados para a formulação.

-Análises de FT-IR indicaram uma efetiva incorporação do fluconazol e timol nas matrizes poliméricas devido a presença de bandas específicas.

-Análises de difração de raios X demonstraram que ambos fluconazol e polímeros encontraram-se em uma forma amorfa após o preparo dos filmes.

-Os filmes vaginais mostraram uma superfície homogênea, uniforme e não porosa durante a análise de microscopia eletrônica. Cristais de fármaco não foram observados.

-Os filmes quitosana/HPMCAS apresentaram maior plasticidade e flexibilidade nos ensaios mecânicos, o que pode explicaria a maior taxa de intumescimento na presença de fluido vaginal simulado.

-Pela análise dos dados de porcentagem de alongamento é possível hipotetizar que a combinação de ácido cítrico e ácido lático atuou como plastificante no sistema.

-A combinação de biopolímeros tornou os filmes mais hidrofílicos, o que facilita o acesso dos fluidos vaginais na formulação e subsequente liberação do fluconazol.

-A presença da quitosana nos filmes contribuiria para o afrouxamento das junções aderentes entre as células epiteliais da mucosa vaginal, explicando as altas taxas de permeabilidade do fluconazol.

-O timol mostrou ser um promotor de absorção químico eficaz, aumentando a distribuição do fluconazol nos tecidos.

-O filme de quitosana e pectina proporcionou maior permeabilidade e retenção do fármaco no tecido. Sugere-se que este resultado pode estar relacionado com a rápida desintegração do filme e baixa capacidade de intumescimento.

-A associação de fluconazol e timol diminuiu a dose efetiva em 50% frente a *C. glabrata*, uma cepa resistente e de alta relevância clínica.

-Filmes de quitosana/HPMCAS deveriam ser priorizados em estudos futuros, pois apresentaram melhores propriedades mecânicas e maior taxa de intumescimento, o que poderia proporcionar uma liberação mais sustentada do fármaco, característica crucial em terapias direcionadas à candidíase resistente.

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