Research Article

A Novel Combination Approach for the Vaginitis Management: Mucoadhesive Chitosan Based Vaginal Films Containing Metronidazole and Nystatin

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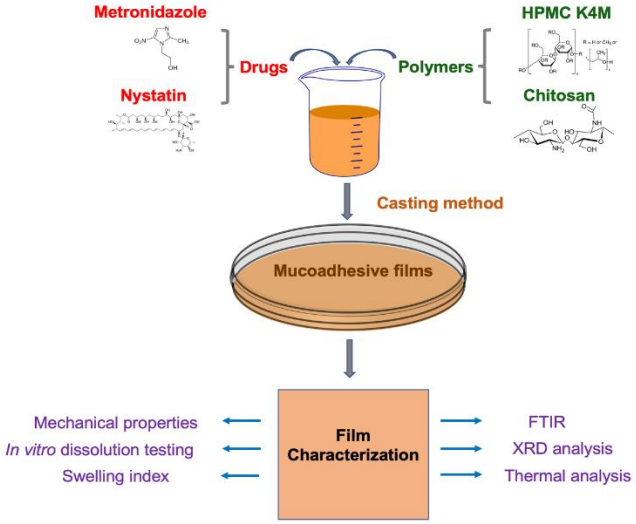
ABSTRACT

Vaginitis is a troublesome by its prevalence and repetition in women. In this study, we developed a novel approach to manage this annoying situation using mucoadhesive chitosan based vaginal films containing metronidazole plus nystatin. Vaginals films were fabricated and measured in terms of thickness, folding endurance, loss on drying, surface pH, weight uniformity, swelling index and drug content uniformity. The morphology of film samples was determined by scanning electron microscopy and the drug crystallinity in the films was assessed by X-ray diffraction and differential scanning calorimetry analysis. In addition, fourier-transformed infrared spectroscopy analysis was also used to investigate the interaction between drugs and excipients. Finally, in vitro antibacterial and antifungal activities of films were evaluated. The results indicated that all films exhibit homogenous surfaces with good mechanical properties. The surface pH of film formulations is in the range of 5-6.5 that favors the vaginal physiology. Thermal analysis and X-ray study showed that metronidazole and nystatin exist in a crystalline form which could ensure the stability of drugs. No interactions between the excipients and drugs could be observed through the FTIR spectra. Interestingly, in vitro drug release profiles revealed a delay pattern and only reached equilibrium after about two hours. The presence of the double combination drug-drug and drug chitosan offered an advantage for fungal and bacterial managements using the fabricated films.

Keywords: Vaginal films, metronidazole, nystatin, vaginitis, antifungal.

GRAPHICAL ABSTRACT



INTRODUCTION

Vaginitis is considered to be one of the most common cause that disturbs women's life and almost all women have to face at least once.[1,2] Vaginitis is caused by several factors. Bacterial vaginosis is the most common cause of vaginitis (40 % to 50 %), while vulvovaginal candidiasis and trichomonal vaginitis contribute approximately 20 % to 25 % and 15% to 20% of cases of vaginitis, respectively.[3] Metronidazole (MET) is an antibiotic that has exhibited antibacterial activity against protozoans such as *Entamoeba histolytica, Giardia lamblia* and *Trichomonas vaginalis*[4] whereas nystatin (NYS) is an antifungal drug, which has been used for the treatment of infection of *Candida* spp[5]. A previous study has revealed that the combination of MET and NYS could prevent vaginal infection better than using MET or NYS alone.[6] The combined products containing MET and NYS has been marketed in different types of dosage forms like ovule or cream with various brand names including Flagystatin, Neo gynoxa, Metrozin® nistatina and Metronist® to treat bacteria (*Trichomonas vaginalis*) and fungi (*Candida albicans*). However, the use of these dosage forms faces some limitations such as drug leakage, inconvenience and poor retention time of drug due to the self-rinsing action of the vaginal tract[7,8] that may be overcome by using bioadhesive vaginal film. In fact, vaginal films showed several promising characteristics like portability, extended retention time, easy application, convenience of storage and handling and increased stability compared to conventional vaginal formulations.[9,10]

For the first time, this study was conducted to develop mucoadhesive films composing of HPMC K4M and chitosan. In this film, chitosan is a cationic natural polymer.[11] It could displays good biological properties such as biocompatibility, biodegradability, availability and nontoxicity.[12,13] In addition, it exhibits antimicrobial activities against some kinds of bacteria and fungi.[14] Furthermore, the use of MET and NYS combination could potential the efficacy in infection management. Physicochemical characterizations of vaginal film formulations loading MET and NYS such as mechanical, swelling and *in vitro* drug release properties were investigated.

MATERIALS AND METHODS

Materials

Metronidazole of pharmaceutical grade (BP 2013) was supplied by Beijing Yibai Biotechnology Co., Ltd. (China) and nystatin of pharmaceutical grade (BP 2016) was supplied by Antibiotice Company (Romania). Chitosan was bought from Shandong Laizhou Highly Bio Products Co., Ltd (China). Hydroxypropyl methylcellulose (HPMC) K4M (Hebei, China) was used. Bovine serum albumin, lactic acid, glucose and urea were purchased from Sigma-Aldrich (UK). Acetonitrile and methanol HPLC grade, glycerin, sodium chloride sodium hydroxide, potassium hydroxide, calcium hydroxide, potassium dihydrogen phosphate, formic acid and acetic acid were purchased from Merk (Germany). Deionized water was purified using a GenPure UV-TOC system (Thermo Scientific, Germany). All other chemicals were of analytical grade.

Methods

HPLC method for simultaneous determination of metronidazole and nystatin

HPLC condition

The HPLC method was developed to measure the concentration of MET and NYS in samples. HPLC Agilent 1260 Technologies (USA) system connected with DAD Detector G1315D. A column Agilent C18 (4.6×250 mm; 5 µm) was used at room temperature. The mobile phase consisted of 0.1% formic acid in water (Solvent A) and acetonitrile (Solvent B) with gradient elution of A:B = 80:20 to 20:80 (0-10 min), 20:80 to 80:20 (10-11 min), 80:20 (11-12 min) at a flow rate of 1.5 mL/min. The detection wavelength was set at 320 nm and the injected volume was 20 µL.

Standard solutions and calibrations

Stock standard solutions (0.5 mg/mL) of each drug (MET and NYS) were separately dissolved in methanol. Then these solutions were diluted with methanol to obtain standard solutions having a range of 1-50 µg/mL for each compound. The calibration curves were constructed by the peak area and the concentration of the drug. The concentrations of MET and NYS in analytical samples were calculated by using a calibration curve.

Preparation of simulated vaginal fluid

Simulated vaginal fluid (SVF) was prepared according to the previous method using the composition shown in **Table 1**. This mixture was adjusted to a pH of 4.2 using HCl, then SVF was used within 5 days and stored at room temperature.

Film preparation

The formulation of films using various polymer ratios was obtained by film casting method. Films contained drugs (metronidazole and nystatin), polymers (HPMC K4M and chitosan) and a plasticizer (glycerin) (**Table 2**).

Table 1: Composition of simulated vaginal fluid

Composition	Concentration (g/L)
Sodium chloride	3.51
Potassium hydroxide	1.40
Calcium hydroxide	0.222
Bovine serum albumin	0.018
Lactic acid	2.00
Acetic acid	1.00
Glycerin	0.16
Urea	0.4
Glucose	5.0

Table 2: Composition of films

Composition	MN1	MN2	MN3
Metronidazole (mg)	200	200	200
Nystatin (mg)	20	20	20
Glycerin (g)	5	5	5
HPMC K4M (g)	0.67	0.80	1.00
Chitosan (g)	0.33	0.20	-
HPMC K4M/Chitosan (w/w)	67:33	80:20	100:0
Purified water	qs to 50 mL	qs to 50 mL	qs to 50 mL

Firstly, HPMC K4M was dispersed in 20 mL of purified water. The chitosan solution was prepared by adding 20 mL of purified water to chitosan, then adjusted to a pH of 4 using 1% acetic acid. Secondly, MET and NYS were dispersed in glycerin, then combined with the chitosan solution. After that, the chitosan solution was dripped over HPMC K4M solution to avoid precipitation by using magnetic stirring at 40 °C. Then, purified water was added to the obtained mixture to 50 mL of solution. Finally, the air bubbles in the resulting solution were removed using a vacuum pump, then poured into a petri dish with a diameter of 8 cm and dried at 40 °C for 72 hours in an oven.

Film characterization

Thickness

The thickness of films was carried out in a digital microscope MT5300L (Meiji, Japan). The films were placed in a holder. The edge of the films was measured and analyzed using imaging software. Three replicate measurements were performed for each film sample.

Folding endurance

The folding endurance of films was conducted by manual folding repeatedly at the same place. The folding endurance value was recorded when the films were broken. The tests were carried out in triplicate.

Loss on drying

The moisture content of the films was evaluated by placing the films in an infrared moisture analyzer (MB 45, Ohaus, Switzerland) at 105 °C for 10 minutes. The percentage of moisture loss was calculated by the difference in weight between the initial weight and the final weight of the film samples.

Surface pH

The surface pH of the films was determined using a pH meter (Sension+ pH 3, Hach, China). The films were contacted with 1 mL of distilled water to swell for one hour at room temperature. Then, the surface pH was measured by touching the electrode with the swelled surface of the films and allowing it to equilibrate for one minute. For each formulation, the measurement was performed in triplicate.

Weight uniformity

Each film was weighed using an analytical balance (Sartorius QUINTIX224 – 1S, Germany). The measurement was repeated three times.

Swelling index

Swelling measurements were performed by immersing an accurately weighed portion of the films (around 50 mg) in simulated vaginal fluid and maintained at 37 °C. At predetermined time intervals (0, 5, 10, 15, 20, 30 and 60 min), the films were carefully removed. The excess of adhering moisture was wiped gently with filter paper and then the films were weighed. The swelling index (SI) was calculated using the weights of the dried (W_o) and swollen (W_t) films as the following equation:

$$SI\ (\%) = [(Wt - Wo)/Wo] * 100$$

Drug content uniformity

The MET and NYS content in each film was evaluated by the HPLC method as described in paragraph 2.2.1. Firstly, 100 mg of each film was homogenized (T18 digital package, IKA, Germany) in 80 mL ethanol 70% and stirred on a magnetic stirrer. Then, ethanol 70 % was added to 100 mL of the mixture in a volumetric and filtered through a 0.45 µm cellulose acetate filter and assayed by the HPLC method. Each film formulation was analyzed in triplicate.

In vitro release studies

Dissolution studies were carried out in a beaker containing 50 mL of simulated vaginal fluid at 37 ± 0.5°C with the hotplate magnetic stirrer C-Mag HS10 S000 (IKA, Malaysia) at a speed of 100 rpm. The films were dispersed in the dissolution media. At 0.5, 1, 2, 4, and 6 hours, 1 mL of the dissolution medium was taken, and another 1 mL of fresh media was added to replace it. The test sample was diluted with methanol and filtered through a 0.45 µm cellulose acetate filter. The amount of MET and NYS released was analyzed by the HPLC method. This study was repeated three times.

Fourier transformed infrared spectroscopy (FTIR)

FTIR analysis of the samples was acquired with a diamond-based ATR accessory (Agilent Cary 630, Agilent Technologies, Malaysia). The scanning was performed with a wave number range from 4,000 to 650 cm^{-1} and a resolution of 2 cm^{-1} at room temperature in absorbance mode.

Differential scanning calorimetry analysis

Thermal analysis of samples was investigated using the differential scanning calorimetry method in a DSC PT1000 Linseis machine (Germany). 5–6 mg of each sample was put into a closed aluminum pan. The scanning temperature range was 40–250 °C with a heating speed of 10 °C/min. During testing, argon gas was maintained at a flow rate of 30 mL/min.

X-ray diffraction analysis (XRD)

The X-ray diffractograms were obtained with a Bruker D8 Advance diffractometer (Germany) operating and equipped with an X-ray tube (Cu- Kα1 radiation: λ = 1.5406 Å, 40 kV and 30 mA). The 2θ range was from 2 ° to 80 ° with a step size of 0.020 ° and step time of 1 s.

RESULTS AND DISCUSSION

Film characterization

Mechanical properties

The film sample showed a smooth surface and uniform texture. The thickness of film formulations shown in **Table 3** was similar, about 0.36 mm. In a previous study, the film thickness ranged from 0.2 mm to 0.7 mm,[15] which could help patients to use it more comfortably than the ovule[16].

Table 3: Physicochemical properties of films (mean ± SD, n=3)

Films	Thickness (µm)	Loss on Drying (%)	Folding Endurance	Surface pH
MN1	367.5 ± 4,4	3.58 ± 0.80	> 100	6.50 ± 0.08
MN2	363.3 ± 6.4	6.05 ± 0.32	> 200	6.26 ± 0.32
MN3	361.5 ± 10.0	4.31 ± 0.23	> 400	5.05 ± 0.29

Folding endurance was investigated to evaluate flexibility and broken resistance of the film. The films must have sufficient resistance to secure mechanical durability in the vaginal application. Films based on only HPMC K4M showed high folding endurance (folded more than 400 times) (**Table 3**). Increasing HPMC K4M content in film formulation causes an increase in film folding endurance, which indicated that this property depends on the concentration of HPMC K4M polymer in the formulation[16].

Loss on drying

The moisture content of films was found to range between 3.58 ± 0.80 and 6.05 ± 0.32 (**Table 3**). The little amount of moisture content in formulations helps them to remain stable and prevents them from being a completely dry and

brittle film. The low moisture content of the films maintain the stability and prevents the formation of dried and fragile films[17].

pH surface

The normal vaginal pH ranges between 3.8 and 5.0, which is moderately acidic.[18,19] The surface pH of all film formulations was observed to range from 5.05 ± 0.29 to 6.50 ± 0.08 (**Table 3**), which favors the vaginal physiology. Furthermore, there could be no irritation between the films and vaginal mucosa.[20]

Weight and drug content uniformity

The uniform of the film's mass was shown in **Table 4**. The weighing films promise the accurate dosage of different films.[21] The drug content of MET and NYS ranged from 97.6 ± 1.4 to 98.5 ± 1.6 and from 97.5 ± 1.3 to 98.2 ± 2.5, respectively (**Table 4**). These results indicated that the drug was dispersed homogenously in the films.

Table 4: Weight and drug content uniformity of the films (mean ± SD, n=3)

Films	Weight (g)	Nystatin content (%)	Metronidazole content (%)
MN1	6.16 ± 0.09	97.5 ± 1.3	98.5 ± 1.6
MN2	6.19 ± 0.19	98.2 ± 2.5	97.6 ± 1.4
MN3	6.21 ± 0.11	97.9 ± 1.4	98.3 ± 2.1

Swelling measurement

The swelling test is important to evaluate the swelling capability of polymers, which provides the presence of vaginal fluid in the films after vaginal placement. The swelling index of the film samples decreased when the amount of HPMC K4M decreased (**Fig. 1**), which suggested that the water uptake of HPMC K4M was higher than chitosan. With an increase in the concentration of HPMC K4M in the film formulations, hydroxyl groups were increased, which could improve the swelling process due to the excellent swelling potential of HPMC.[22,23]

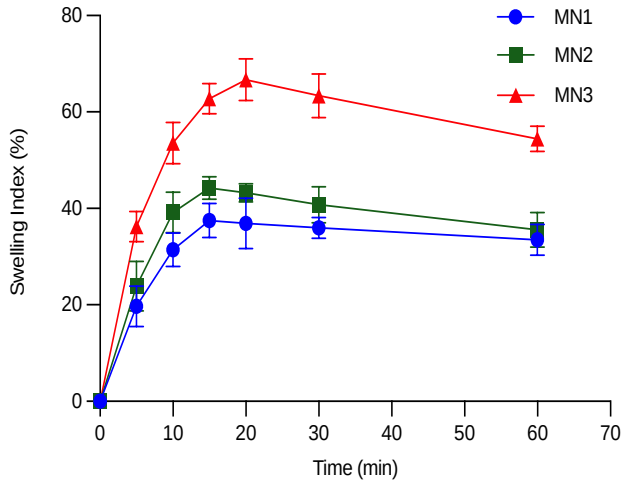


Figure 1: Swelling index of various films

Spectroscopic data

The FTIR spectra of MET and NYS raw, HPMC K4M, chitosan and the films were shown in **Fig. 2**. The IR spectrum of MET showed several characteristic peaks at 3221 cm^{-1} and 3098 cm^{-1} (O-H stretching); 1536 cm^{-1} (N-O stretching); 1431 cm^{-1} (OH deformation); 1264 cm^{-1} (O-H in plane deformation); 1186 cm^{-1} (C-O stretching); 1074 cm^{-1} (C-H in plane bending); 905 cm^{-1} and 824 cm^{-1} (C-H out of plane bending)[24]. The characteristic peaks of NYS at 3374 cm^{-1} (O-H stretching); 2938 cm^{-1} (C-H2 stretching); 1693 cm^{-1} (C=O stretching); 1623 cm^{-1} (C=C asymmetric stretching); 1576 cm^{-1} (C=C stretching) and 1057 cm^{-1} (C-O stretching)[25,26]. The HPMC K4M spectrum showed at a range of 3050–3700 cm^{-1}, which was attributed to the O–H stretching. The peaks at 1025 cm^{-1} and 980 cm^{-1} were related to -CO and CH- groups[27]. The chitosan spectrum showed absorption bands at 2883 cm^{-1}; 1447 cm^{-1}; 1335 cm^{-1}; 1090 cm^{-1} and 900 cm^{-1} which were characteristic of C-H stretching, O-H stretching, C-O stretching, C-O-C stretching and N-H_2 stretching vibrations, respectively. Comparing the FTIR spectrum of HPMC K4M, the film samples showed similar peaks, suggesting the presence of a large amount of HPMC K4M loaded on the film formulations. This result could illustrate the absence of interactions between drugs and excipients.

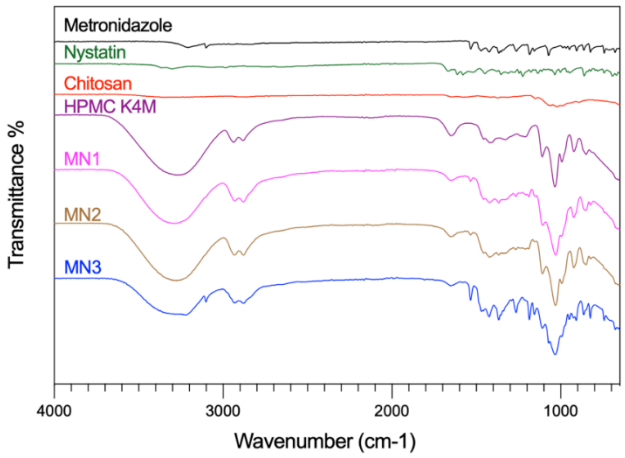


Figure 2: FTIR analysis of materials and films

Thermal analysis

DSC analysis can be seen in **Fig. 3**. The NYS raw showed an endothermic peak at 173.9 °C[26], which indicated that NYS material could exist in a semicrystalline state[28]. DSC of pure MET revealed a sharp melting peak at 160.3 °C,[29-31] associated with high crystallinity. When MET and NYS were loaded on the films, the endothermic peak of these drugs disappeared. The absence of an endothermic peak of MET and NYS could be due to the dilution effect of HPMC K4M and chitosan in the films.

XRD analysis

The crystallinity of MET raw material was observed through an XRPD pattern in Fig. 4. The MET raw showed sharp diffraction peaks at 12.28°, 13.84°, 22.28°, 24.06°, 24.72°, 28.12°, 28.70°, 30.02° and 34.70°. The

diffractogram of MET was in agreement with the results reported in previous studies.[32,33] On the other hand, diffraction peaks of NYS at 11.13°, 13.85°, 16.36° and 20.41° were identified, which revealed that NYS raw displayed a crystalline substance.[34] No diffraction peaks were observed in the X-ray patterns of HPMC K4M and chitosan, which could associate with the amorphous nature of polymers[35].

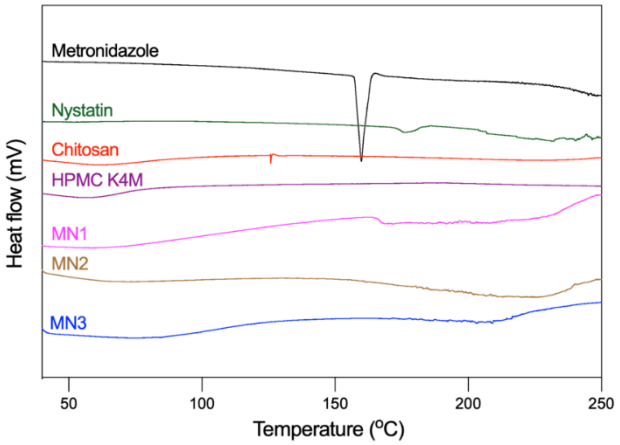


Figure 3: DSC thermograms of drugs, polymers and films.

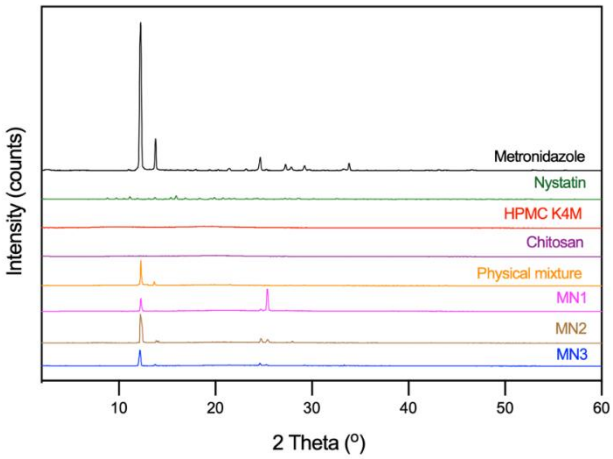


Figure 4: XRD analysis of materials and films

The X-ray study of the films showed that the reduction in peaks corresponding to crystalline MET was due to the effect of the excipient dilution.[16] This confirmed the hypothesis that the crystalline form of MET may remain the same. Meanwhile, the X-ray analysis of the films did not present any peak regarding NYS raw material, which could be explained by due to the small amount of NYS in film formulations.

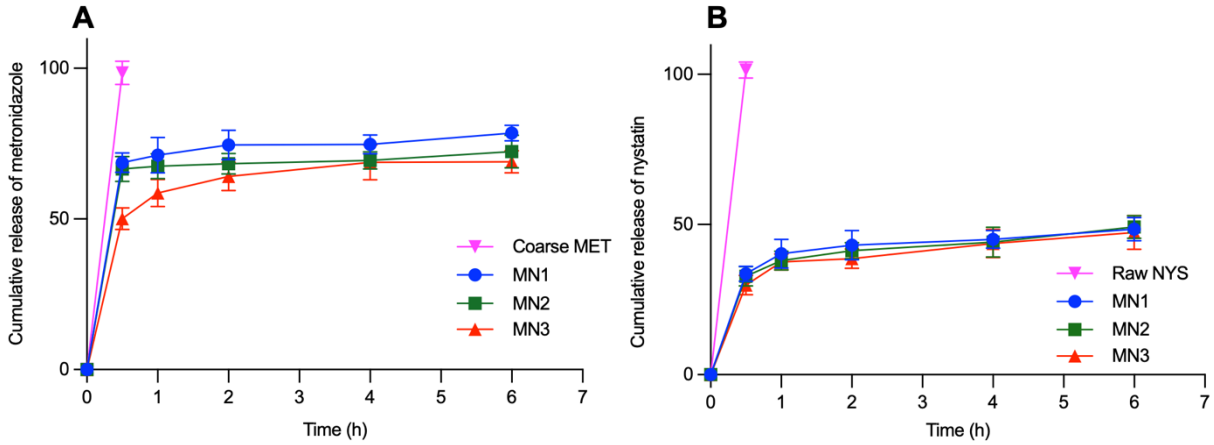


Figure 5: Released percentage of metronidazole (A) and nystatin (B) from using with different polymer ratios

In vitro drug release studies

The effect of variable HPMC K4M and chitosan content on the percentage of drug release was observed and the results were shown in **Fig. 5**. Formulations containing variable amounts of chitosan indicated that as chitosan content was gradually increased from 0 to 0.33 g, MET and NYS release percentages increased from 29.8% to 33.6% and from 50.1% to 68.7% after 1 hour, respectively. The film based on only chitosan showed the highest drug release, which could be due to the higher solution viscosity of HPMC K4M. As HPMC K4M and chitosan absorbed water from vaginal fluid, the viscosity of the HPMC K4M solution was greater than that of the chitosan solution. This led to lower drug release from the gel. After approximately 2 hours, the MET and NYS release reached the equilibrium value.

CONCLUSIONS

For the first time, this study revealed the potential of the development of mucoadhesive films composing of HPMC K4M and chitosan for vaginitis management. Furthermore, the use of MET and NYS combination could potential the efficacy in controlling infection. The films were fabricated and characterized for the physicochemical properties. The folding endurance could ensure the endurance of films whereas thickness and pH of films should favor the application and biocompatibility of films. Importantly, these films could take advantage of mucoadhesive characteristic of HPMC K4M to maintain drug concentration at site and the anti-microbial properties of chitosan for better treatment. Altogether, using combination of metronidazole and nystatin and dual-polymers HPMC K4M and chitosan

sustained drug release and potential films as a promising approach to treat vaginitis.

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