

Formulation And Characterization of Carageenan Gels Encapsulating Amphotericin B And *Lactobacillus acidophilus* Against Candidal Vaginitis

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Abstract The present study is concerned with the development and characterization of bioadhesive carageenan gel encapsulating Amphotericin B and *Lactobacillus acidophilus*, prepared by graft co-polymerization against Candidal vaginitis. Intravaginal gel systems based on bioadhesive polymer (carrageenan) were characterized with respect to swelling index, bioadhesive strength, percent encapsulation and *in vitro* drug release antimicrobial studies. A marked increase in swelling index of gel encapsulating *Lactobacillus* was found to be 1.9 ± 0.35 . The percent encapsulation of drug was found to be $98.63\% \pm 0.2\%$ and that of *Lactobacillus* was 91.81 ± 0.01 . The viability was observed for interval of 6 hrs on trypton soya agar and showed that viability was highly conserved till 4 hrs. The antimicrobial study of gels encapsulating Amphotericin B and *Lactobacillus* showed that carageenan gel can inhibit *Candida albicans* upto a maximum extent. Bioadhesivity study also conducted for gels that showed a bioadhesivity of $84.66\% \pm 0.5\%$ with drug, $88.66\% \pm 0.02\%$ with *Lactobacillus*. *In-vitro* drug release showed a sustained type release of drug from the polymer i.e. there was initial burst of Amphotericin B up to 5 hours, after which there was a sustained release upto 10 days. Thus it has been concluded from the present study that bioadhesive gels encapsulating Amphotericin B can act as promising drug carriers along with *Lactobacillus* against candidal vaginitis.

Keywords: Candidal Vaginitis, Carageenan, Swelling index, Encapsulation, Bioadhesivity

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1. INTRODUCTION

Candidal Vaginitis (candidiasis) is a typical problem caused by *Candida albicans* which is characterized by inflammation of the female genitalia. Vaginitis is very common and is reported by as many as 75% of women at some point in their lives. It is one of the most common forms of *Candida* infection in women. These fungi can be found all over the body and are normally present in moist areas of body. Donati and Di Vico (2010) has reported that 20%-50% of all women normally carry yeast in the vagina without the presence of symptoms [1].

The vaginal flora is the dynamic system mainly consisting of *Lactobacillus* (Doderlein's *bacilli*) which is the most prevalent organism in vaginal environment together with many other facultative and obligate aerobes and anaerobes (*Cornebacteria* and yeast). The *Lactobacilli* produce sufficient lactic acid to acidify vaginal secretions to a pH 3.5 - 4.5. An imbalance in the intestinal flora where good bacteria are killed due to excessive use of antibiotics, unhealthy lifestyles and a weak immune system usually cause over growth of yeast. Various drugs which are used to cure candidal vaginitis are Amphotericin B, Fluconazole, Nystatin, metronidazole, Itraconazole, clotrimazole etc. Traditionally solutions, suppositories, foams and tablets have been used as vaginal formulations. Amphotericin B is one of the effective drugs used against candidal vaginitis which is a macrocyclic polyene with antifungal activity. It is associated with ergosterol, forming a transmembrane channel that leads to monovalent ion leakage causing fungal death. The drugs has various side effects like irritation, diarrhoea, headache, hypersensitivity, low availability of drug, poor patient compliance, and systemic side effects due to higher total dose and high frequency of drug administration. Sustained release drug delivery systems offer a promising approach in overcoming these drawbacks The use of mucoadhesive polymers for development of gels may help in controlled release of bioactive agents. Hydrogels are hydrophilic polymer networks which have the capability to imbibe water or biological fluids. They can exhibit dramatic changes in their swelling behaviour, network structure, permeability and mechanical strength in response to change in pH or ionic strength of surrounding fluid or temperature Gupta (2012) [2]. Probiotics offer alternative anticandidal approach as they lower the pH and help in restoring the balance of microflora. Probiotics are living micro organisms that favourably influence the health of the host by improving the indigenous microflora. Boskey et al. (2001) has confirmed that *Lactobacilli* play an important role in the inhibition of growth, adhesion and spread of pathogenic microbes and thus possess good antimicrobial properties [3] The present study is an endeavour to explore formulation and characterization of carageenan

based hydrogels encapsulating amphotericin B and probiotics as effective drug delivery systems against *Candidal vaginitis*.

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2. MATERIALS AND METHODS

Commercially available IP injection of “Amphotericin B” under the brand name of Fungitericin manufactured by Gufic biosciences limited, Kabilpore, Navsari, Gujarat state. (India) was used, Sporolac manufactured by Uni Sankyo Ltd. containing around 150 million spores of *Lactobacillus sporogenes* in 1g sachet was used in the study, Carrageenan, tryptone soya agar, dialysis membrane manufactured by HiMedia was used in the study, Lyophilized culture of *Candida albicans* (MTCC-183) was procured from IMTECH, Chandigarh, India. All the reagents of standard grade were used in the study.

2.1 Preparation of Carrageenan Based Vaginal Gel Hosseinzadeh (2010)[4]

1g of kappa-carrageenan was dispersed in 30ml of distilled water. The dispersion was mixed using a magnetic stirrer for 20 minutes and then placed in a water bath at 80 °C for 20 minutes. After dissolving κ- carrageenan, the initiator Ammonium per sulphate (0.01g in 5 ml of distilled water) and Methylene bis acrylamide (0.05g in 5 ml of distilled water) were simultaneously added. The mixture was again stirred on a magnetic stirrer for 20 min. The monomer acrylamide (1.0g) was added and gelation was observed after 15 min. 1 ml of the drug Amphotericin B and 500mg spores of *Lactobacillus* was added to the 10 ml of gel and mixed thoroughly (By graft co-polymerisation).

2.2 Physicochemical Characterization of Gels Encapsulating Amphotericin B and *Lactobacillus*

2.2.1 Determination of pH of vaginal gel

1g of gel was dissolved in 30 ml of distilled water and the electrode was then dipped in to gel formulation and constant reading was noted. The measurements of pH of each formulation were repeated three times Ramadan (2008) [5].

2.2.2 Swelling Index Studies

Swelling index is the measure of intensity of the gelation of the gel. More the swelling index, tighter the gel is and vice-versa. 1g of gel was taken on a filter paper and then placed in a beaker containing SVF for 6 hours. Samples were removed from beakers at different time intervals (30 min., 60 min, 180 min, 360 min.) Samples were put in a dry place for some time after that these were reweighed at different time intervals as optimized by Pal and Nayak (2011) [6].

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Swelling Index was calculated using:

$$Sw\% = [(W_t - W_0) / W_0] \times 100$$

Sw = Equilibrium percent swelling

W_t = weight of swollen gel after time 't'

W_0 = original weight of gel at zero time.

Where, T = amount of total drug, C=drug present in supernatant

2.2.3 Determination of encapsulation efficiency of gel

To 1g of each vaginal gel 10ml of methanol was added in a volumetric flask followed by continuous shaking for 10 minutes and then diluted to 100 ml with phosphate buffer (pH=7.4) and centrifuged. The supernatant was filtered. The absorbance of filtrate was taken at 416 nm⁵.

$$\text{Percent Encapsulation} = \frac{\text{Total amount of drug} - \text{amount of drug present} \times 100}{\text{Total drug}}$$

2.2.4 *In vitro* Drug Release Studies

The *in vitro* release of drug was evaluated in phosphate buffer. For this the cellophane membrane was equilibrated with simulated vaginal fluid (SVF) overnight. *In vitro* release studies of Amphotericin B loaded vaginal gels was carried out for 10 days at room temperature by suspending dialysis bag (prepared from cellophane membrane) containing drug loaded gel in phosphate buffer. After regular intervals of 1h, 2h, 3h, 4h, 5h, 6h, 24hrs, 1day, 2days, up to 10 days, 1 ml of supernatant was taken out and replaced with same amount of freshly prepared phosphate buffer. The sample from supernatant was taken in a quartz cuvette and diluted with 3 ml of distilled water. Absorbance was taken at 416 nm and concentration of drug was calculated from standard curve. The same procedure was followed to carry out *in vitro* drug release studies for *Lactobacillus*. Absorbance was taken at 340 nm.

Viability of *Lactobacillus* from the drug released *in vitro*:

100µl of phosphate buffer in which vaginal gels (containing Amphotericin B, combination of Amphotericin B with *Lactobacillus*) were suspended was taken at regular interval of 1 hour, 2 hours upto 6 hours and spread on trypton soya agar plates. The plates were incubated at 37°C for 24-48 hours.

Bioadhesivity testing by *in vitro* wash off method:

The mucoadhesive properties of vaginal gels were evaluated by modified method of Patel et al. (2013) [7]. A fresh excised piece of goat intestine mucosa (2.5cm x 2.5cm) was mounted on a glass slide. The gel sample (1g) was placed over the intestine mucosa and another slide with 5g of

weight was applied over it for 5 minutes. Then the prepared specimen was transferred to the beaker placed at an angle of 60° from the surface beneath. The phosphate buffer from the burette at a constant flow rate of 2ml/min was added to the specimen. The test was carried out until the gel was removed from the mucosal surface.

For each of three vaginal gels, samples loaded with Amphotericin B, *Lactobacillus* alone, Amphotericin B and *Lactobacillus* in combination were developed to study the antimicrobial activity of the gels. Blank samples and free drug alone served as control. Supernatant of *lactobacillus* 100µl was added to well in a petriplates. The plates were incubated at 25°C for 24 hours. The study was continued for 48 hours for the blank samples so as to check the biodegradability of the crosslinked gel.

RESULTS

In the current study we aimed at developing drug delivery systems based on natural polymer carageenan encapsulating Amphotericin B and *Lactobacillus* against *Candidal vaginitis*. Bioadhesive gel systems were characterized based on pH, swelling index and percent encapsulation.

Physicochemical characterization of Carageenan gels encapsulating Amphotericin B and *Lactobacillus*

pH: pH of vaginal gels was estimated by mixing 1g of gel in 30 ml distilled water as optimized by Ramadan (2008)[5] with the help of pH meter [Table 1]:

Table 1: pH of carageenan gels encapsulating Amphotericin B, *Lactobacillus* and their combinations.

S.No.	Vaginal gels	pH (Mean + S.D)
1.	Carageenan - Amphotericin B	7.2+ 0.10
2.	Carageenan - <i>Lactobacillus</i>	6.75 + 0.005
3.	Carrageenan - Amphotericin B + <i>Lactobacillus</i>	7.01 + 0.02

From above values it was seen value of pH increases in case of carageenan gels encapsulating drug alone. But in case of *Lactobacillus* values decreases.

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This is because of the effect of the *Lactobacillus* which is normal flora of vagina and tends to decrease the pH of the environment.

Percent Encapsulation of Carageenan Gel:

Amount of drug encapsulated in different gel systems was calculated as shown in Ramadan (2008) [5]. For this, to 1g of each vaginal gel 10ml of methanol was added in a volumetric flask followed by continuous shaking for 10 minutes and then diluted to 100ml with phosphate buffer (pH=7.4) and centrifuged at 5000 rpm for 10 minutes. The supernatant was filtered. The absorbance of filtrate was taken at 416 nm as shown in [Table 2].

Table 2: Percent encapsulation of drug (Amphotericin B) and *Lactobacillus*.

S.No.	Gels	Encapsulation Efficiency Mean \pm S.D
1.	Carageenan – Amphotericin B	98.63 \pm 0.23
2.	Carageenan - <i>Lactobacillus</i>	91.81 \pm 0.01

Swelling Index:

Swelling index is the measure of intensity of the gelation of the gel. More the swelling index, tighter the gel is. As the swelling values of vaginal gels increases, the rate of drug release also increases [Figures 1, 2 and 3].

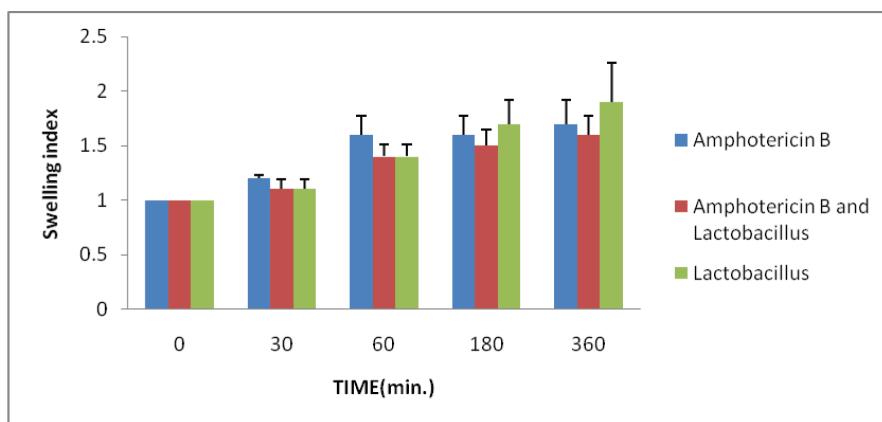


Figure 1: Swelling index of the Carageenan based vaginal gel encapsulating drug/probiotic.

Amphotericin B

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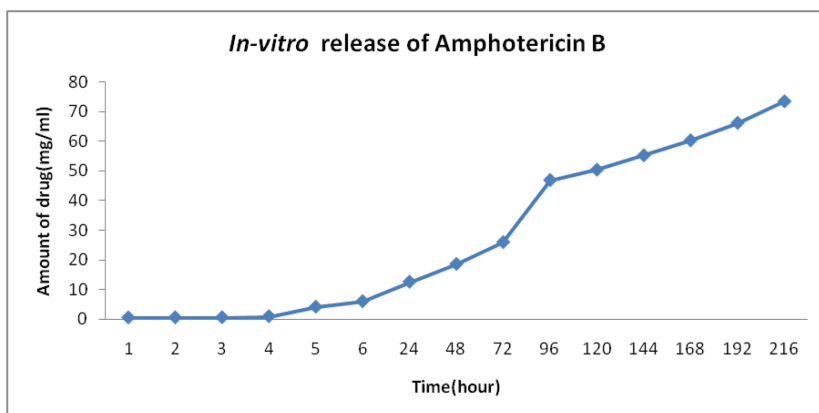


Figure 4: *In vitro* drug release from carrageenan gel.

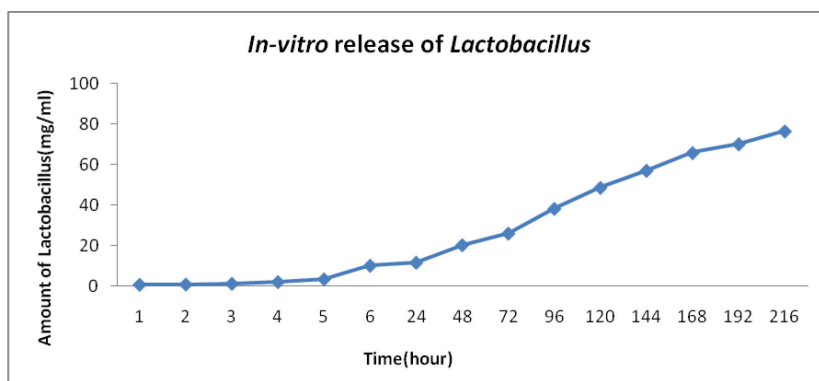


Figure 5: *In vitro* release of *lactobacillus* from carrageenan gel.

Viability of *Lactobacillus* in carageenan gels:

Viability of *Lactobacillus* was assayed in vaginal gels for 6 hours [Table 1]. 100 μ l of phosphate buffer in which vaginal gels (containing both Amphotericin B and *Lactobacillus*) were suspended, was taken at regular interval of 1 hour, 2 hours up to 6 hours and spread on trypton soya agar plates. The plates were incubated at 37 $^{\circ}$ C for 24-48 hours and examined.

The viability study of *lactobacillus* was even studied in vaginal gels as was done with gels encapsulating Carageenan gel. The viability was observed for interval of 6 hours on trypton soya agar. It was seen that viability conserved high viability till 4 hours but then it started decreasing.

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Table 3: Viability of *Lactobacillus* in carrageenan gels was shown for different time intervals.

Time Interval (in hrs)	Carrageenan gel
1	+++
2	++
3	++
4	++
5	+
6	+

30×10^6 cfu/ml was given '-', $30-150 \times 10^6$ cfu/ml was given '+' and $>150 \times 10^6$ cfu/ml was given '++'.

Bioadhesive strength of vaginal gels:

Bioadhesion implies the attachment of a drug carrier to a specified location study was done to see the adherence of drugs and lactobacillus to the epithelial cells of vagina. As the bioadhesivity increases, rate of drug release also increases as shown in [Table 4].

Table 4: Bioadhesive strength of carrageenan gels.

S.No.	Vaginal gel used	Biadhesive Strength Mean \pm S.D
1.	Carageenan – Amphotericin B	84.66 \pm 0.5
2.	Carageenan – <i>Lactobacillus</i>	88.66 \pm 0.2

***In vitro* drug release from carrageenan gel:**

The *in vitro* release of drug was evaluated in phosphate buffer. For this the cellophane membrane was equilibrated with simulated vaginal fluid (SVF) overnight. *In vitro* release studies of Amphotericin B loaded vaginal gels were carried out for 10 days at room temperature by suspending dialysis bag (prepared from cellophane membrane) containing drug loaded gel in phosphate buffer. The same procedure was followed for *Lactobacillus*.

Percentage of drug released up to 24 hrs from carrageenan gel = 12.55%

Percentage of drug released upto 10 days from carrageenan gel = 73.38 %

Lactobacillus:

Percent of lactobacillus released upto 24 hrs from carageenan gel = 11.41%

Percent of lactobacillus released upto 10 days from carageenan gel = 78.42 %

DISCUSSION

Candidal vaginitis is an infection of vagina which is characterized by inflammation of female genitalia. The causative agent is yeast *Candida albicans*. Amphotericin B is one of the effective drugs used against candidal vaginitis. Probiotics is another strategy to use with the drugs against vaginitis as they lower the pH and helps in restoring the balance of microflora in vagina as demonstrated by Gil et al. (2010) [8]. There are two modes of drug delivery for the treatment of candidal vaginitis-oral and vaginal. Presently marketed formulations include oral (tablets), suppositories, vaginal rings and creams. Vaginal drug delivery presents several advantages: ease of administration, hepatic first pass-effect bypass, low systemic drug exposure and increased permeability for some drugs when comparing to the oral or the other routes. Low enzymatic activity may also benefit drug delivery through vaginal route Merabet et al. (2005) [9]. In the current study intravaginal gel systems based on bioadhesive polymers like sodium alginate, chitosan, carrageenan were developed and characterized.

Several polymers including natural (collagen, carageenan, sodium alginate, chitosan, lectin etc.) and synthetic (lactic acid based polylactic-co-glycolic acid and polyanhydride, polyorthoesters, polycynoacylates, polycaprolactone) have been employed for formulating sustained release vaginal drug delivery system. Natural polymers e.g. carrageenan, chitosan and alginate have proved better over the synthetic polymers because of better bio compatibility, non toxicity and enhanced bioavailability.

In our present study natural polymers have been chosen for sustained drug delivery system against *Candidal vaginitis*. Carrageenan gel was prepared by graft co-polymerization of *kappa*-carrageenan, using ammonium per sulphate as a free radical initiator in the presence of methylene bisacrylamide as a cross linker. This hydro gel system exhibit a high sensitivity to pH and serves as a platform to a wide range of pharmaceuticals. Due to sensitivity for pH, it showed several changes of swelling and deswelling , which contributes to its suitability for controlled drug release.

The prepared bioadhesive gels showed pH near to the neutral pH, which indicates that none of the formulation will cause irritation in vaginal mucosa as reported by Sudeendra et al. (2010) [10].The surface pH of all formulations ranged from pH 4.89 to 7.52 as seen in [Table 1]. The vaginal gel containing

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lactobacillus alone formulations shows the pH away from the neutral pH as seen in [Table 1]. *Lactobacillus* does not change the environment of the vagina, but it does interact with the environment to maintain normal conditions. *Lactobacillus* produce lactic acid, which help the vagina maintain a normal acidic pH. It works to try and prevent any other microbes from altering its pH by producing a balance of lactic acid to counter any intruding microbes. It has been reported in literature that *Lactobacillus* bacteria are also acidophilic (acid-loving), and therefore easily tolerate the acidic pH of the vagina. Their optimum pH range for growth is from pH=3.5 to pH=6.8. *Candida albicans* grows well between pH=5.1 and 6.9. It is important to note that *Candida albicans* can survive a low, acidic pH environment (pH=2), but its growth is inhibited under such acidic conditions. Hence pH play important role in treatment of *Candidal vaginitis*.

Swelling index is the measure of intensity of the gelation of the gel. More the swelling index, tighter the gel is and vice-versa. As the swelling of polymer increase, drug release rate also increase. Our study depicts the difference between the swelling index of all vaginal gels encapsulating in carageenan, chitosan and sodium alginate polymers. It was seen from graphs that swelling index values increases with time and swelling index was more in case of carageenan and chitosan gels as compared to sodium alginate gels. According to the comparison of the corresponding swelling profiles of formulations with/without *lactobacillus*, it could be seen that the *lactobacillus* resulted in a marked increase in swelling rate. Increased value in swelling index means gel formed is tighter and hence will result in improved drug release. Dobaria et al. (2009) have reported that the swelling capability of polymer is crucial for its bio adhesiveness as adhesion occurs shortly after the swelling ^[11]. Swelling is important for the assessment of adhesion. After swelling, adhesion does occur, but with a weak bond formed. The phenomenon of swelling increasing can be explained by the good disintegration effect of *Lactobacillus*, which made gels increase in volume and construct porous channels on surface and inside of gels. The porous channels increased the area of contacting between polymer particles and water so that the polymers could be hydrated more easily.

Percent encapsulation was observed best with Amphotericin B as compared with *Lactobacillus* in the vaginal gels. It depends upon interaction and encapsulation of hydrophilic drug with hydrophobic polymer. [Table 2] shows comparative percent encapsulation of Amphotericin B of carageenan, sodium alginate and chitosan gels are 98.63 ± 0.23 , 98.86 ± 0.4 and 98.82 ± 0.3 respectively. It has been reported by Dhakar et al. (2012) that encapsulation efficiency depends on solubility of drug and solvent in continuous phase [12].

Viability of *Lactobacillus* was observed to check the adhesion of *Lactobacillus* to vaginal epithelial cells. It was seen [Table 3] that viability of *Lactobacillus* was maximum till 4 hours and decreases at 6 hour. *Lactobacillus* viability was highly conserved till 4 hours but then it started decreasing with time.

In vitro drug release studies were carried out in phosphate buffer under simulated vaginal conditions through a cellophane membrane. A faster release initially indicates that the drug in the solution in the space outside the gel matrix initially diffuses quickly. The release of drug within the gel is controlled by both the nature and concentration of polymer used. The bioadhesion in carrageenan may be due to the presence of more negative charges in the form of sulphate and carboxylic ions which help in attachment of gel to the mucus membrane more strongly as shown by Bhowmik and Nayak (2009) [13]. In our present study drug release kinetics of different vaginal gels showed a sustained type release of drug from the polymer i.e. there was initial burst of Amphotericin B up to 5 hours, after which there was a sustained release upto 10 days.

Bioadhesion is the formation of a bond by interfacial forces in which one surface is biological in nature. The bioadhesive strength of all vaginal gels [Table 4] results depict that vaginal gel formulation with both drug and *Lactobacillus* showed maximum strength.

Amphotericin B and *Lactobacillus* encapsulated in carrageenan gel showed bioadhesive strength 84.66 ± 0.5 and 88.66 ± 0.2 respectively. Other workers explained that Bioadhesion is a very important aspect for maintaining high drug levels at the site of administration and prevents expulsion of formulation. By maintaining effective drug concentration for longer time, one can achieve successful eradication of infectious agent.

CONCLUSIONS

Carrageenan systems were found to have maximum bioadhesive properties that help in delaying vaginal transit of encapsulated compounds. The pH of vaginal gels was found to be less when the *Lactobacillus* was encapsulated in the carrageenan gel. The percent encapsulation was best with the Amphotericin B drug of vaginal gels (carrageenan- 98.63 ± 0.23). Bioadhesivity was observed best with Carrageenan gels. The viability for *Lactobacillus* in the gel was high till for 4 hours. Antimicrobial studies of gels encapsulating Amphotericin B and *Lactobacillus* showed that carrageenan gel can inhibit *Candida albicans* upto a maximum extent as

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compared to other two gels chitosan and sodium alginate. Thus it has been concluded from the present study that bioadhesive gels can act as promising therapeutic agents against candidal vaginitis.

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Nil

Conflicts of interest

There are no conflicts of interest

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