INTRODUCTION

Dysbiosis is the result of disturbance of vaginal microbiota and is a major cause of vaginal infections. Vaginal infections such as, bacterial vaginosis, candidiasis and trichomoniasis are very common gynaecological conditions prevalent in women of reproductive age group (Mulu et al., 2015). Another common vaginal infection is aerobic vaginitis which is mainly caused by disturbance of lactobacilli flora and overgrowth of pathogenic aerobic microflora including, E. coli, S. aureus etc (Donders et al., 2011). Most of these vaginal infections increase susceptibility to sexually transmitted infections or other pathogens, causing mixed infections. The primary line of treatment for aerobic vaginitis often includes oral antibiotics combined with topical treatment of broad-spectrum antibacterial like Metronidazole. Although a variety of conventional dosage forms such as tablet, pessary and cream are reported for local treatment, leakage and poor retention of formulation due to self-cleansing action of the vagina, are the major limitations of these dosage forms. Mucoadhesive formulations allow interaction of formulation components with a vaginal mucosal layer for a prolonged period, increasing the contact period and efficacy of the formulations (Menard, 2011). Various approaches are
reported in order to increase drug solubility, including cyclodextrin complex formation solid dispersions or some chemical modifications. But there are various limitations associated with these approaches (Kaparea et al., 2017).

Chitosan is an extensively explored cationic polymer for its mucoadhesive property. It is obtained by partial deacetylation of chitin and reported to be biocompatible and biodegradable. Chitosan is also reported to increase the drug permeation by the paracellular transport mechanism. Although an excellent mucoadhesive polymer, chitosan is soluble in acidic pH and therefore has a lower ability to retard or control the drug release in the vaginal acidic environment due to rapid disintegration. To overcome this problem, one of the emerging trends in the formulation of polyelectrolyte complexes of chitosan with oppositely charged polymer. Polyelectrolyte complexes (PEC) are the association complexes formed between oppositely charged particles such as a polycationic polymer (chitosan) with anionic polymers, carrageenan (Abruzzo et al., 2013). PEC is a suitable carrier for controlled release of drug with good mucoadhesive properties and lower pH dependence. Carrageenan is an anionic biopolymer reported for the use in the vaginal cavity due to its antimicrobial activity for preparation of sexually transmitted pathogen. In most cases, PEC between chitosan and the anionic polymer is prepared in solution first followed by removal of the solvent by lyophilisation. The dry PEC is then further used for the preparation of dosage form. However, this process is complicated and lengthy with a low yield. In order to avoid this drawback, few studies have reported the concept of in situ PEC wherein anionic and cationic polymers are compressed into tablets and in situ PEC are formed due to ionization of these polymers in a biological fluid (El-Kamel et al., 2002).

Considering this in the present study, an attempt has been made to formulate in situ PEC based tablet using chitosan as cationic polymer and carrageenan as an anionic polymer for vaginal delivery of Metronidazole.

**MATERIALS AND METHODS**

Materials
Metronidazole was provided as gift sample by Hindustan Antibiotics Ltd, Pune, Chitosan (100 kDa) and Carrageenan (CG, GP 209NF) were purchased from Himedia Ltd (Mumbai), Microcrystalline Cellulose (MCC) and Isopropyl alcohols were purchased from Loba Chemie (Mumbai), Magnesium Stearate and Talc were purchased from Research Lab (Mumbai). All other chemicals were of analytical grade.

**Preparation of mucoadhesive tablets of metronidazole**

The muco adhesive vaginal tablets of metronidazole were prepared using polymers, chitosan and carrageenan and their combination at 1:1 molar ratio (Table 1). The metronidazole tablets were prepared by wet granulation method in which granules were prepared by alcohol, followed by compression to tablets. For the preparation of granules, weighed quantities of drug and polymers were taken and mixed thoroughly. The solution of Poly (vinyl) pyrrolidone K 30 (PVP K30) as a binder was made in isopropyl alcohol. This solution was added to the previously mixed powder blend to obtain a wet mass. The wet mass was passed through 18 mesh sieve to obtain granular mass. These granules were then dried at 55°C in a vacuum oven and again dry passed through 18 mesh sieve. After blending with lubricant and glidant, the granules were then compressed on a rotary tablet machine (Rimek Mini Press MT-II) with 12 mm punch to obtain 500 mg tablets. These tablets were then subjected to different evaluation tests (Prado et al., 2008).

**In vitro drug release study**

The tablets were evaluated for in vitro drug release using USP dissolution apparatus II (Veego, VDA-8D4). The dissolution was carried out in 900 ml of citrate phosphate buffer pH 5.2 with rotating paddle at 50 rpm and at 37± 0.5 °C. After every one hour, aliquot of 1 ml was removed from each flask, filtered by whatmann filter paper and diluted up to 10 ml with buffer. The sample was analyzed for the quantity of drug released at 319.5 nm using UV visible double beam spectrophotometer (Shimadzu, UV1701, Japan). The dissolution medium was replenished with an equal volume of the fresh medium after each removal.

**Swelling behaviour of the matrix tablet**

Previously weighed tablet (T1) was placed in a petri dish containing 5 ml of citrate phosphate buffer pH 5.2. After every 1 hour interval, the tablet was removed from the dish and weight of the tablet was noted (T2) after removal of excess water present on the surface of the tablet. The per cent water uptake was calculated by the following formula,

\[
\text{Per cent water uptake} = \frac{(T_2 - T_1) \times 100}{T_1}
\]

Where T1 is the initial weight of the tablet, T2 is the final weight of swollen tablet (Kuunisto et al., 2011). The experiment was repeated for six tablets (n = 6).

**Lyophilization**
Table 1: Composition of polymer and PEC tablet of Metronidazole

| Formulation Code* | Metronidazole (mg) | Excipients (mg) | Chi-
|                  |                   | Car-    | MCC | PVP | Mg | Talc |  |
|                  |                   | rageenan |    |     |    |      |  |
|                 |                   |                  |   |     |    |      |  |
| CS1              | 250               | 150       |    | 65  | 25 | 5    | 5 |
| CS2              | 250               | 200       |    | 15  | 25 | 5    | 5 |
| CG1              | 250               |           | 150| 65  | 25 | 5    | 5 |
| CG2              | 250               |           | 200| 15  | 25 | 5    | 5 |
| PEC              | 250               | 47.84     | 152.16 | 15 | 25 | 5    | 5 |

* Avg weight of each tablet formulation was 500 mg

Table 2: Antimicrobial efficacy of formulation after 6 hrs and 24hrs of incubation

| Sr.no | Incubation Time (hrs) | E. coli Control | Marketed formulation (Flagyl) | Placebo | PEC |
|-------|-----------------------|-----------------|-------------------------------|---------|-----|
| 1     | 00 hrs                | 6±0.025         | 5 ± 0.006                     | 5.954±0.002 | 5.845±0.008 |
| 2     | 06 hrs                | 6.963±0.007     | 3.544 ± 0.012                 | 5.939±0.006 | 3.633±0.011 |
| 3     | 24 hrs                | 7.146 ± 0.05    | NG                            | NG      | NG |

The tablet formulations of chitosan (CS2), carrageenan (CG2) and PEC tablet in 1:1 molar ratio (PEC) were allowed to swell in 5.2 pH citrate phosphate buffer for 24 h and then refrigerated for 24 h followed by lyophilization. The lyophilized products were characterized by FTIR, SEM and DSC for the presence of any interaction.

**FTIR Spectra interpretation**

The IR spectra of previously lyophilized formulation of chitosan (CS2), carrageenan (CG2) and PEC tablet in 1:1 molar ratio (PEC) were recorded by FTIR spectrometer (Shimadzu, FTIR-8400s, Japan) with diffused reflectance. The baseline correction was made using dried potassium bromide (KBr). The powdered sample was mixed with potassium bromide to obtain a uniform dispersion. The dispersion was kept in the sample cell which was fitted on the sample holder and the spectrum was recorded. The spectra were used to identify the major functional groups and to determine any possible interaction of the formulation components.

**Differential scanning calorimetry (DSC)**

The physical state of metronidazole loaded chitosan, carrageenan and PEC tablet were studied using differential scanning calorimetry on a Perkin Elmer 4000 DSC system. The sample (1 mg) was placed in Al pan and sample analysis was performed under nitrogen purging at a flow rate of 20ml/min using empty Al pan as reference. The DSC spectra were recorded from range, 30-300 °C at a rate of heating, 10 °C /min.

**Morphological Analysis by scanning electron microscopy (SEM)**

The external morphology of lyophilized formulations of chitosan (CS2), carrageenan (CG2) and PEC tablet (PEC) was studied by SEM. The powdered sample was sprinkled on Al stubs using double adhesive tape. Prior to estimation, samples were coated with 20 nm thin platinum layer by auto fine coater to render them electrically conductive. The coated samples were scanned after placing them in SEM chamber. The photomicrographs were obtained at accelereation voltage of 10 kV and morphology was studied.

**In-vitro muco adhesion strength measurement by using Brookfield texture analyzer**

In vitro mucoadhesion study of tablet, formulations were performed against goat vaginal mucosa using a texture analyzer (Brookfield, CT3 Texture analyzer, USA). Goat vaginal mucosa was collected from slaughter house and after appropriate washing was rapidly frozen to −20 °C. Before testing, the mucosal membrane was thawed at room temperature. The mucosa was hydrated with 5.2 pH citrate phosphate buffer and was attached to base of texture analyzer. To the other moving arm of texture analyzer, a tablet to be tested was attached using adhesive tape. The arm with tablet was lowered and contact of a tablet
Antimicrobial study

The antimicrobial activity of the PEC formulation (PEC) was evaluated against E. coli ATCC 11105. E. coli was grown aerobically in Lysogeny broth medium at 37 °C for 24 h. Viability of E. coli in citrate phosphate buffer (pH 5.2) was compared with the viability of the respective bacterium cultured in the presence of developed vaginal tablet. Briefly, microbial suspension prepared from a broth culture of E. coli was added to Erlenmeyer flasks containing 100 ml of citrate phosphate buffer saline (pH 5.2). The final strength of E. coli culture is adjusted in solution as 6 logs of CFU/ml. Before the addition of the formulations, T₀ reading was taken. To each flask, various tablet formulations were added and incubated aerobically at 37°C. Microbial counts (viable) were taken at 0, 6 and 24 h by using LB agar plates and resulting CFU/ml were reported and compared against control and standard (Abruzzo et al., 2013).

RESULTS AND DISCUSSION

In vitro drug release study

Drug release from dosage form reported to be occur through some common mechanisms that include erosion, diffusion and degradation mechanisms (Kapare et al., 2020). Formulations CS₁, CS₂, CG₁ and CG₂ are formulations without PEC and showed the immediate release of drug due to bursting of tablet in dissolution media. The sustained release tablets of Metronidazole with PEC (1:1) demonstrated 99.9±3.8 % drug release after 8h of dissolution study (Figure 1). Chitosan being cationic polymer tends to demonstrate strong ionic interactions with negatively charged polymer or surfaces. Carrageenan, owing to its anionic nature, interacts with positively charged surfaces. In case of carrageenan, interactions responsible are hydrogen bonding and an open expanded conformation. Poly-electrolyte complexes are formed due to strong electrostatic interaction between cationic chitosan and anionic carrageenan. These complexes dissociate

with mucosa was established for 10 sec at 10 g force. The probe was then removed from mucosa at a rate of 5mm/s. The force required to detach the tablet from mucosa was measured as mucoadhesive force (g) (Takayama et al., 1990).

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in the presence of water causing quicker hydration of the polymer matrix and resulting in swelling of the polymer chains. This results in hydrated three-dimensional gel network in a tablet that restricts diffusion of the drug. The drug release kinetics study revealed diffusion controlled drug release from PEC tablet, thus following Higuchi matrix model.

**Swelling behaviour**

Figure 2 indicates the water uptake of polymer and PEC tablet over time. The amount of water uptake in case of tablets with polymer alone was higher, resulting in higher swelling of tablet in the initial two h of study. After 2 to 3 h, the tablets were disintegrated due to weaker gel structure of polymers when used alone. PEC tablets demonstrated shower hydration in the initial hours of study, followed by greater swelling after 6 hours of study. Chitosan/carrageenan complex presented ionic interactions between positively charged chitosan and negatively charged carrageenan and exhibited swelling behaviour due to dissociation of the complex. The mutual repulsion between charges of chitosan and the charges of carrageenan, as well as the entry of water with counter ions to neutralize these charges, causes swelling.

**Spectral Characteristics**

The FTIR spectrum of carrageenan showed a broad absorption band in the range of 1050.0 to 1150.0 cm⁻¹ assigned to –SO₄²⁻ groups (Figure 3). The FTIR spectrum of chitosan showed an intense &
Figure 4: DSC of A. CS2, B. CG2, C. PEC

Figure 5: Scanning Electron Microscopy of (A) Lyophilized CS2 (A-X: 1000). (B) Lyophilized CG2 (A-X: 1000). (C) Lyophilized PEC (A-X: 1000)
broad absorption band at 1654.98 cm\(^{-1}\) assigned to the asymmetrical bending vibration of \(-\text{NH}\) group & absorption band at 1525.74 cm\(^{-1}\) representing symmetrical bending vibration of \(-\text{NH}\) group. Because of protonation of amino group (+\(\text{NH}_3^+\)) it results into decreased intensity of peaks. Absorption band was slightly shifted to 1658.8 cm\(^{-1}\) for asymmetrical bending vibration and 1519.3 cm\(^{-1}\) for symmetrical bending vibration of amino group. Presence of absorption band at 3097.7 cm\(^{-1}\) & 2953.1 cm\(^{-1}\) indicates \(\text{NH}\) stretching vibration of protonated amino group. Thus, the presence of protonated amino group confirmed the formation of polyelectrolyte complexes at the tablet surfaces in the presence of dissolution medium.

**Differential Scanning calorimetry**

Differential Scanning calorimetry (DSC) study was carried out for Lyophilized Tablet formulation of CS2, CG2, and PEC. The DSC results are shown in Figure 4. All three tablet formulation showed a sharp endothermic peak at 161.3°C. Which indicates melting point of drug Metronidazole. Presence of sharp peak indicates the crystalline nature of the drug. For CS2, a broad endothermic peak was observed around 80 °C and another endothermic peak was observed 263.8°C whereas, for carrageenan formulation CG2, exothermic peak was observed at 268.2°C. Both the sample showed a peak in a similar range. These exothermic peak indicated degradation of CS2 and CG2. According to (Shao et al., 2014) formation of in situ PEC can be indicated by degradation peak of polymer in the DSC. But, no obvious interaction between polymer was indicated by the DSC curve of PEC. The peak at 271°C was visible but was very weak. This indicates the possibility of formation of in situ PEC at the surface of tablets.
Morphological Analysis by SEM

Figure 5 indicates SEM of lyophilized CS tablet, CG tablet and PEC tablet. Lyophilized chitosan formulation revealed fibrous threadlike structure, whereas lyophilized carrageenan formulation showed the swollen platelike structure of carrageenan. PEC formulation exhibited a network-like structure with more surface roughness at higher magnification.

In-vitro mucoadhesion strength measurement with Brookfield Texture Analyzer

The presence of chitosan or carrageenan in the formulations influenced significantly the tablet mucoadhesion properties (Figure 6). This behaviour could be due to the ionisation of amino groups of chitosan at pH 5.2. The cationic charge on chitosan is responsible for interaction with the negatively charged carrageenan and sulfate residues of mucin glycoprotein. Mucoadhesive properties of carrageenan could be attributed to its hydrogen bonding and an open expanded conformation. Force of adhesion for PEC on Goat Vaginal Mucosa using Brookfield Texture Analyzer was found to be 1.2 g/cm² and CS2 and CG2 on Goat Vaginal Mucosa using Brookfield Texture Analyzer was found to be 0.6g/cm² and 0.9 g/cm². This indicates higher mucoadhesive strength of PEC as compared to CS2 and CG2.

Antimicrobial Study

Antimicrobial efficacy of the PEC was tested by direct incubation of the formulation in the bacterial culture (E. coli) of known concentration ($1 \times 10^6$ CFU/ml) and then checking the viability of the E. coli in the presence of formulation (Table 2, Figure 7). The antimicrobial efficacy of formulation was compared with marketed Metronidazole tablet (Flagyl) and Placebo formulation containing chitosan and carrageenan. The results indicated that there was a significant decrease in the microbial count of marketed ($T_{6}; 3.542$ log CFU/ml) and developed PEC formulation ($T_{6}; 3.63$ log CFU/ml) after 6 hrs of incubation. The formulations without drug did not reduce the microbial count significantly ($T_{6}; 5.93$ log CFU/ml).

After 24h of incubation, all the formulations including placebo exerted strong antimicrobial activity, decreasing the microbial count below the detection limit. Placebo formulations also indicated antimicrobial activity which might be due to the presence of chitosan and carrageenan, which are reported having antimicrobial activity. Per cent reduction in the microbial count was 49%, 47.8% and 14.7% with marketed formulation, PEC and placebo after six h of incubation. After 24h of incubation 100% reduction was observed by all the formulation indicating strong antimicrobial activity. Statistical analysis was carried out by one way ANOVA followed by Dunnett’s multiple comparison test where $P < 0.01$ implies significance.
CONCLUSIONS

Muco adhesive metronidazole tablet formulation was designed and developed with combination with polyelectrolyte complex as a novel approach for vaginal drug delivery system. Developed formulations were characterized for various formulation parameters and antimicrobial activity which revealed desirable characteristics and good antimicrobial potential. This drug delivery system showed promising outcomes and thus, it can be further developed as a novel dosage form for the improved therapeutic potential of metronidazole for application in vaginal drug delivery system.

Acknowledgement

Authors are thankful to Hindustan Antibiotics Ltd, Pimpri, Pune for providing the Metronidazole as gift samples.

Funding Support

The authors declare that they have no funding support for this study.

Conflict Of Interest

All authors declare that they have no competing interest for this study.

REFERENCES

Abruzzo, A., Bigucci, F., Cerchiara, T., Saladini, B., Gallucci, M. C., Cruciani, F., Vitali, B., Luppi, B. 2013. Chitosan/alginate complexes for vaginal delivery of chlorhexidine digluconate. Carbohydrate Polymers, 91(2):651–658.

Donders, G. G. G., Bellen, G., Rezeberga, D. 2011. Aerobic vaginitis in pregnancy. BJOG: An International Journal of Obstetrics & Gynaecology, 118(10):1163–1170.

El-Kamel, A., Sokar, M., Naggar, V., Gamal, S. A. 2002. Chitosan and sodium alginate—Based bioadhesive vaginal tablets. AAPS PharmSci, 4(4):224–230.

Kapare, H. S., Sathiyarayanan, L., Arulmozhi, S., Mahadik, K. R. 2020. Caffeic Acid Phenethyl Ester Loaded Poly (ε-caprolactone) Nanoparticles for Improved Anticancer Efficacy: Formulation Development, Characterization and in Vitro Cytotoxicity Study. Nanomed Res J, 5(4):324–331.

Kapare, H., Sathiyarayanan, L., Arulmozh, S., Mahadik, K. 2017. Design and Development of Indian Propolis Loaded Poly (ε-Caprolactone) Nanoparticles For Improved Anticancer Efficacy. International Journal of Pharmaceutical Research, 9(3):73–80.

Kaunisto, E., Marucci, M., Borgquist, P., Axelsson, A. 2011. Mechanistic modelling of drug release from polymer-coated and swelling and dissolving polymer matrix systems. International Journal of Pharmaceutics, 418(1):54–77.

Menard, J. P. 2011. Antibacterial treatment of bacterial vaginosis: current and emerging therapies. Int J Womens Health, 3:295–305.

Mulu, W., Yimer, M., Zenebe, Y., Abera, B. 2015. Common causes of vaginal infections and antibiotic susceptibility of aerobic bacterial isolates in women of reproductive age attending at Felegehirwot referral Hospital, Ethiopia: a cross sectional study. BMC women's health, 15(1):42–42.

Prado, H. J., Matulewicz, M. C., Bonelli, P., Cukierman, A. L. 2008. Basic butylated methacrylate copolymer/kappa-carrageenan interpolyelectrolyte complex: Preparation, characterization and drug release behaviour. European Journal of Pharmaceutics and Biopharmaceutics, 70(1):171–178.

Shao, Y., Li, L., Gu, X., Wang, L., Mao, S. 2014. Evaluation of chitosan-anionic polymers based tablets for extended-release of highly water-soluble drugs. Asian Journal of Pharmaceutical Sciences, 10(1):24–30.

Takayama, K., Hirata, M., Machida, Y., Masada, T., Sannan, T., Nagai, T. 1990. Effect of interpolymer complex formation on bioadhesive property and drug release phenomenon of compressed tablet consisting of chitosan and sodium hyaluronate. Chem. Pharm, 38(7):1993–1997.