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TERBINAFINE INCORPORATED SODIUM HYALURONATE MICROSPHERE MUCO-ADHESIVE SYSTEM FOR VAGINAL CANDIDIASIS

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Keywords:

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ABSTRACT: The aim of the present investigation was to prepare and evaluate novel bioadhesive vaginal microspheres containing Terbinafine (TBH) in order to provide long-term therapeutic activity at the site of infection and prove the same using *in vitro* anti fungal activity of the same using *Candida albicans*. Microspheres were prepared by the Solvent extraction technique using sodium hyaluronate and Arlacel A. Microspheres were characterized by SEM, DSC, FTIR, particle size analysis and evaluated for percentage yield, drug loading, encapsulation efficiency and *in vitro* drug release. FTIR and DSC studies showed that no chemical changes or alterations occurred in the drug and polymers. The sphericity factor indicated that the prepared microspheres were spherical. Formulation (TBHMs) indicated a controlled *in vitro* drug release and good bioadhesive strength. The *in vitro* anti fungal activity confirmed for a controlled and prolonged capacity of the prepared novel formulation. The results indicated that this drug delivery system can be explored for controlled intra-vaginal drug release.

INTRODUCTION: At an average 75% of women will have at least one episode of VC, and 40% - 45% will have two or more episodes [1, 2]. Vaginal candidiasis is evaluated to be the second most cause for vaginitis after bacterial vaginosis. *Candida albicans* represents 85% to 90% of cases [3]. Major risk factors causing VVC include sexual activity, continuous antibiotic usage, pregnancy, and immunosuppression caused by uncontrolled HIV infection or diabetes [4, 5].

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Over 20% of women may have yeast as part of their natural vaginal microbiome and the majority will be asymptomatic [6]. The majority of vaginal candidiasis cases are caused by *Candida albicans*, but non - albicans species were also usually associated with recurrent or chronic forms of diseases [7-9]. Non - albicans species are usually less sensitive to azole antifungal [7, 10, 11].

Several investigators believe that an increasing in vaginal infection due to non-albicans, (*C. glabrata*, *C. tropicalis* and *C. dubliniensis*) has been observed during recent years [10, 12]. Reports show that azole resistance was detected among some *Candida* species; especially *C. glabrata* isolates [13, 14]. In addition long term use of antifungal drugs can cause recurrent vaginitis [15].

In the past few decades, considerable attention has been focused on the development of the Novel drug delivery system (NDDS) for natural herbal drugs. Developing nano-dosage forms (polymeric nanoparticles and nanocapsules, Microspheres, liposomes, solid lipid nanoparticles, phytosomes and nanoemulsion etc.) have a number of advantages for herbal drugs, including:

- Enhancement of solubility and bioavailability,
- Protection from the toxicity,
- Enhancement of pharmacological activity,
- Enhancement of stability,
- Improving tissue macrophages distribution,
- Sustained delivery and
- Protection from degradation by physically and chemically.

So the novel drug delivery systems of drugs have a potential future for increasing the activity and overcoming problems associated with using drugs as such [16-21].

Mucoadhesion is a topic of current interest in the design of drug delivery systems. Mucoadhesive microspheres exhibit a prolonged residence time at the site of application with improved absorption and facilitate an intimate contact with the underlying absorption surface and thus contribute to improved and / or better therapeutic performance of drugs [22]. Many such mucoadhesive microsphere formulations using various drugs have been developed for oral, buccal, nasal, ocular, rectal and vaginal routes for either systemic or local effects [23].

Apart from local effective drugs the vagina also provides as a promising site for systemic drug delivery, because of its large surface area and rich blood supply [24]. The active agents meant for the systemic delivery when given by this route of administration bypass the hepatic first pass metabolism. In addition, a prolonged contact of a delivery system with the vaginal mucosa may be achieved more easily than at other absorption sites like the rectum or intestinal mucosa. However, despite all the advantages of a vaginal application, changes in the membrane during the menstrual cycle and postmenopausal period are major challenges [25-27]. Quite often, the delivery systems suffer from migration within the vaginal/rectal lumen, which might affect the delivery of the active agent to the specific location (target site) [28]. The use of mucoadhesive polymers for the development of delivery system helps in increasing the migration of the drug to target site, thereby promoting better therapeutic efficacy [29]. The polymers used in the development of vaginal and rectal delivery systems include mucin, gelatin, polycarbophil and poloxamer [30-32]. These polymers are able to swell rapidly when placed in aqueous environment and therefore exhibiting a controlled drug release [34-36].

Vaginal drug delivery systems include a large variety of pharmaceutical forms such as semi-solids, tablets, capsules, pessaries, liquid preparations, vaginal films, vaginal rings, foams, and tampons. Most widely used semi-solid preparations for vaginal drug delivery include creams, ointments, and gels [37, 38]. The main advantages of semi-solid preparations are acceptability, feasibility, and low cost. On the other hand, messiness, discomfort, and leakage are its main disadvantages [39].

In fact, one of the problems presented by conventional vaginal drug delivery systems is rapid removal from the application site [40]. Gels are semi-solid systems comprising small amounts of solid, dispersed in relatively large amounts of liquid, yet possessing more solid-like character [41]. These systems form a three-dimensional, polymeric matrix in which a high degree of physical (or sometimes, chemical) reticulation has been comprised [42]. They are formed of long, disordered chains that are connected at specific points and the connections are reversible however the molecular mechanisms of gelation are poorly understood [43]. Gels can present several advantages over other vaginal drug delivery systems such as higher bioavailability, safety, versatility, and economical savings [44]. It is well known that the choice of dosage formulation can influence the disposition of an active substance [45].

The major problem in treating patients with *Candida vaginitis* is that this organism develops resistance to topical and systemic azoles so we are going for novel formulations such as vaginal gel for better therapy [46]. Terbinafine hydrochloride is one of the fungicidal allylamine groups of drugs with broad spectrum of antifungal activity. It interferes with fungal sterol biosynthesis at an early stage.

It also inhibits squalene epoxidation, leading to intracellular accumulation of toxic squalene responsible for fungal cell death [47]. Oral dosage of Terbinafine mainly led to Hepatotoxicity, hematological problems, drug interactions, and systemic side effects [48]. Instead of repeated and frequently application of topical creams, it is anticipated that Terbinafine hydrochloride topical gel would be more efficiently used for the complaince of the patients [49, 50]. Hyaluronic acid is a glycosaminoglycan and part of the extracellular matrix. Relative to its molecular weight, it can bind huge amounts of water making it a promising ingredient with good moisturizing effect and high hydrating properties. The polymer contains a derivative of hyaluronic acid (sodium hyaluronate), which maintains the biocompatibility and interactivity of hyaluronic acid, and also adequate hydration of mucosa. Mucosal administration of the formulation prepared using this polymer has good adhesiveness to the vaginal mucosa and long acting hydration thus improving the dry state of the vagina. It also improves the spontaneous recovery of small lesions that are caused by friction of vagina [51, 52]. The objective of this article is development and evaluation of mucoadhesive microspheres using terbinafine and carry out the *in vitro* anti-fungal activity for the developed formulation (TBHMs).

MATERIALS: Terbinafine HCl (TBH) was a gift sample from (Hetero labs, Hyderabad, India), India. Sodium hyaluronate with a molecular weight of approximately 1300 kDa [63, 64] was a gift sample from Kumar organic products Ltd., Bangalore, India and Arlacel A was obtained from Sigma-aldrich, Bangalore, India. Sabouraud Dextrose Agar was purchased from Himedia chemicals Ltd, Mumbai, India. All other chemicals and reagents used were of analytical grade. Millipore water was utilized for all the studies. The organism was obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India. All other reagents were of analytical grade and used without further purification.

Preparation and Characterization of Sodium Hyaluronate - Terbinafine HCl Microspheres:

Preparation of Microspheres by Solvent Extraction Method: Microspheres of sodium hyaluronate were prepared according to solvent extraction method [53]. According to this method mentioned above, different quantities of polymer concentrations were altered till better encapsulation efficacy was obtained. Sodium hyaluronate was dissolved in DMSO at a concentration of 6% w/v [68]. Drug concentration (at 1% w/v) of TBH [65-67] in DMSO was added to the polymer solution and the mixtures were stirred for 20 min at 1000 rpm using a mechanical stirrer (RW20, IKA WERK Instruments). The polymer/TBH mixture was added to mineral oil containing 0.5% w/v Arlacel A at a ratio of 1:16 v/v [53]. An emulsion was formed by stirring with a disperser (KINEMATICA) at a rate of 10000 rpm for 10 min [68].

Ethyl acetate, at a ratio of 2:1 v/v, was quickly added to extract the DMSO and mineral oil and to precipitate microspheres of sodium hyaluronate/ TBH. The suspended microspheres were filtered under pressure (1.5atm) through a polyamide membrane using a steel filter fitted with a magnetic stirring shaft. The powder was resuspended twice in ethyl acetate and then twice in n-hexane to remove the excess mineral oil and surfactant. The microspheres were then dried under vacuum for 24 hr. The influence of some parameters such as polymer type and concentration, oil phase volume, surfactant concentration and stirring speed were slightly modified and used [68].

Micromeritic Properties: Angle of repose is the maximum angle possible between the surface of a pile of microspheres and the horizontal plane. Fixed funnel method was employed [54]. Apparent bulk density was determined by pouring the samples in bulk into a graduated cylinder. Tapped density was determined by placing a graduated cylinder containing a known mass of powder on a mechanical tapper apparatus (ETD-1020, Electrolab, India). Samples were tapped until no further reduction in volume of the sample was observed. Carr's index was calculated [54].

Compatibility Studies:

Fourier Transform Infrared Spectroscopy (FTIR): Potassium bromide was mixed with the sample, compressed into a pellet and spectra were taken between 400–4000 cm^{-1} using an FTIR spectrophotometer (FT-IR-8400-S, Shimadzu, Japan).

Differential Scanning Calorimetry (DSC): About 2mg of sample was placed in an aluminium pan and hermetically sealed. DSC thermograms were recorded from 0 to 200 °C. The instrument was calibrated using high purity indium metal as standard. Dynamic scans were taken in a nitrogen atmosphere at the heating rate of 5 °C min^{-1} (DSC-60 Shimadzu).

Particle Size Analysis and Sphericity of Microspheres: Particle size analysis of microspheres was carried out by optical microscopy. About 200 microspheres were selected randomly and their size was determined using an optical microscope (SK-6, Suswox Optic, India) fitted with a standard micrometer scale. To determine sphericity, tracings of TBH microspheres (magnification 45×) were taken on black paper using a camera lucida (Model Prism type, Rolex, India). Sphericity factor (S) was calculated as:

$$S = \frac{P^2}{12.56 \times A}$$

Where A is the area (cm^2) and P is the perimeter (cm) of the circular tracing [55]. Scanning electron microscopy (SEM) photographs were taken using a scanning electron microscope (Joel-LV-5600, Japan) at room temperature. Samples were fixed on a scanning electron microscope sample holder with a double-sided adhesive tape and coated with a layer of gold of 1.5×10^{-10} m for 2 min using a sputter coater (Edwards 3-150 Å, England) in a vacuum of 30.4 kPa of argon gas. Photographs were observed for morphological characteristics and to confirm the spherical nature of microspheres.

Drug Loading and Encapsulation Efficiency: Microspheres (100mg) were extracted with 5mL of methanol, diluted with pH 4.5 citrate-phosphate buffer, filtered and analyzed for drug content after suitable dilution, at 282 nm [56].

***In vitro* Drug Release Studies:** Release studies were carried out on prepared formulations in triplicate, employing a basket type dissolution tester-USP XXII (TDT-08L, Electrolab, India) using 600mL of pH 4.5 citrate phosphate buffer as dissolution medium at 100 rpm at 37 ± 0.5 °C to mimic the vaginal conditions [57-59]. Five ml of the sample was withdrawn at different intervals and analyzed by the UV method at 282 nm [56].

***In vitro* Antifungal Studies:** Antifungal activity was evaluated by the cup-plate method using Sabouraud Dextrose Agar plates inoculated with *Candida albicans*. A volume of 20 mL of sterilized agar media was dispersed into a sterilized Petri dish and allowed to solidify. Each Petri dish was divided into three sectors, and a bore (6mm) was made in each sector using a sterile cork borer. Each bore in a different sector was loaded with a placebo polymer (negative control), terbinafine pure drug (positive control) and microspheres loaded TBH. Petri dishes were incubated at the temperature of 37 ± 0.5 °C for 24 hr to allow the growth of microorganisms. The zone of inhibition produced by the microspheres loaded TBH towards the organism was measured (mm).

RESULTS AND DISCUSSION: In the present study, microspheres were prepared using various polymers. Solvent extraction method was optimized by using sodium hyaluronate and Arlacel A to entrap the drug. Being a water insoluble drug, TBH could be entrapped into water insoluble polymers by the solvent extraction method.

Polymer sodium hyaluronate was selected for microsphere preparation because it is slightly soluble in aqueous media but is permeable, have the ability to produce pH-independent drug-release profiles and have release rate controlling ability, non-toxicity, non-irritancy, stability at vaginal pH and compatibility with the drug [60].

The viscosity of the polymer solution was found to be a critical parameter that influenced the dispersion and the shape of the polymer droplets in the surrounding oil phase. Consequently, changes in drug concentration and polymer caused variations in the size and physical characteristics of the microspheres. Other process parameters, such as rotor speed of the disperser and the time required for the addition of ethyl acetate to the emulsion influenced the aggregation of the polymer droplets and the formation of microspheres. Indeed, for the production of large batches of microspheres, a high rotor speed was employed to obtain sodium hyaluronate / TBH microspheres within the desired size range.

Furthermore, the addition of ethyl acetate to the polymer-mineral oil emulsion was performed as quickly as possible to avoid polymer aggregation. The flow property of microspheres was studied by calculating the angle of repose (q in degrees) and compressibility index (CI, %). The obtained data along with related parameters are presented in **Table 1**. The values of q ranged from 31.5 to 35.2° indicating that the microspheres had good flow properties. The CI value was found to be in the range of 19.4 to 25.3 %, which also indicated good flow properties. All the data in **Table 1** implies that TBHMs V is a better formulation with good micromeritic properties.

TABLE 1: EVALUATION PARAMETERS OF TBH MICROSPHERES

Formulation (TBHMs)	TBH (mg)	Sodium hyaluronate (mg)	Arlacel A (mg)	Size (µm)[a]	Θ(⁰)[a]	CI (%)[a]	Tapped density (gcm^{-3})[a]	Yield (%)[a]	Actual drug loading (%)[a]	Encapsulation efficacy(%)[a]
I	100	100	8.3	33.2±0.4	30.5±0.31	25.4±0.2	0.345±0.003	57.1±0.2	19.6±0.4	77.6±0.7
II	100	200	16.6	44.5±0.3	32.7±0.35	22.4±0.2	0.412±0.008	62.3±1.2	22.5±0.4	82.5±0.7
III	100	300	25	46.7±1.2	34.8±0.43	24.6±0.1	0.389±0.005	73.1±1.4	27.9±0.7	66.8±0.6
IV	100	400	33.3	49.2±0.9	34.6±0.40	23.9±0.4	0.336±0.007	51.2±1.0	24.2±0.9	81.3±0.4
V	100	500	41.6	53.2±0.4	31.5±0.31	19.4±0.2	0.263±0.006	77.4±0.2	29.6±0.4	88.6±0.7
VI	100	600	50	59.2±1.4	30.7±0.59	19.0±0.1	0.261±0.004	75.4±0.2	24.6±0.1	86.7±0.1

a Mean ± SD, n = 3.
Sodium hyaluronate and Arlacel A quantities are in the ratio 12:1 [53, 68]

FTIR Studies: The FTIR spectra of pure TBH and microsphere formulation (TBHMs V) are reported in **Fig. 1**. Positions of peaks in FTIR spectra of pure TBH were compared with the spectrum of TBH containing microspheres. Characteristic IR absorption peaks of pure TBH of the aromatic C-H stretch (3040 cm^{-1}), aromatic C=C stretch (1474 cm^{-1}) and aromatic C-H bending (777 cm^{-1}) were also present in the spectrum of the TBH loaded microspheres. Peaks at wavelengths corresponding to the pure drug were also observed in the microsphere formulation (TBHMs V). The FTIR spectra of the pure drug and formulation (TBHMs V) indicated that the positions of characteristic peaks of TBH were not altered after their successful entrapment in the microspheres, suggesting the absence of changes or alterations in the drug and other components of the formulation (TBHMs V) [61].

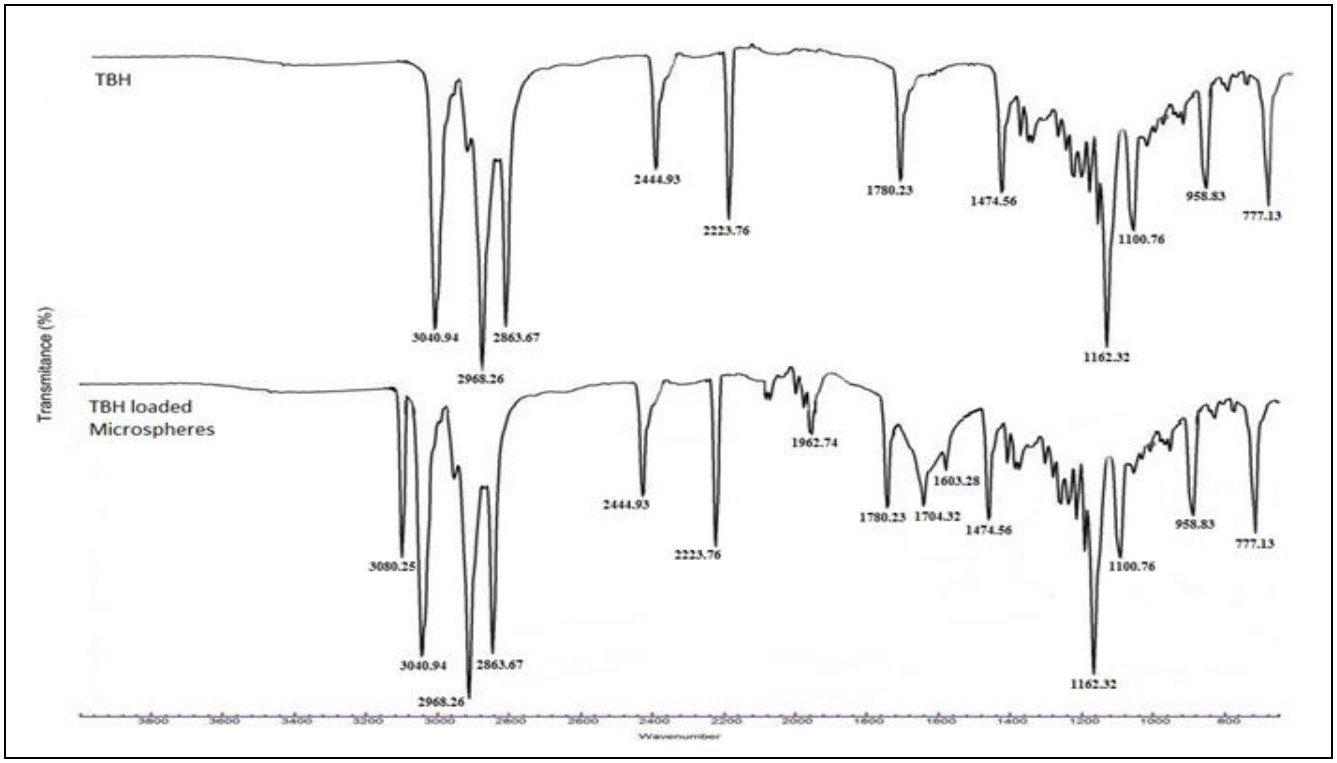

FIG. 1: FTIR SPECTRA OF PURE DRUG TERBINAFINE AND MICROSPHERES FORMULATION (TBHMS V)

TABLE 2: INTERPRETATION OF FTIR OF DRUG (TBH) AND MICROSPHERES FORMULATION (TBHMS V)

Description	Characteristic peaks
TBH	3040.94, 2968.26, 2863.67, 2444.93, 2223.76, 1780.23, 1474.56, 1162.32, 958.83 and 777.13
Sodium hyaluronate	3060,3080.25,2968.26,2940.12, 2916.23, 1962.74, 1780.23, 1704.32, 1603.28, 1474.56, 1162.32, 1000-1100
Formulation (TBHMs V)	3060, 3080.25, 3040.94, 2968.26, 2940.12, 2916.23, 2863.67, 2444.93, 2223.76, 1962.74, 1780.23, 1704.32, 1603.28, 1474.56, 1162.32, 958.83 and 777.13, 1000-1100

Differential Scanning Calorimetry (DSC): DSC thermograms of pure drug and drug loaded microspheres are shown in **Fig. 2**.

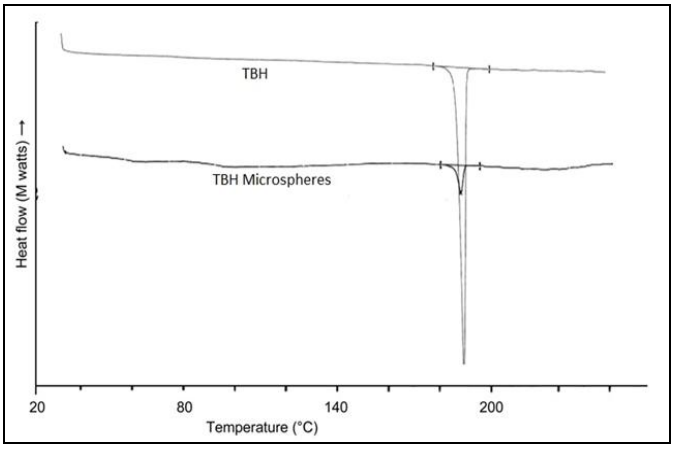

FIG. 2: DSC SPECTRA OF PURE TERBINAFINE AND MICROSPHERES FORMULATION (TBHMS V)

The DSC thermogram of pure TBH showed a sharp melting endotherm at 198.47 °C. This melting endotherm was also observed for formulation (TBHMs V) at 197.58 °C, indicating the absence of drug and polymer changes. However, the melting endotherm of formulation (TBHMs V) was not as sharp as that of pure TBH, which may be a result of the presence of polymers and the change in heat capacity of the polymer as it undergoes transition from the glassy to liquid state during microsphere formation [62].

Particle Size: In general, the size of microspheres (TBHMs V) ranged from 18 to 60µm. Particle size increased with an increase in polymer concentration and particle size decreased with a decrease in polymer concentration. This can be explained by the fact that at higher polymer concentration, the viscosity of polymer solution increased, thereby producing bigger droplets during emulsification, which were later hardened due to the evaporation of solvents.

SEM and Sphericity: SEM photographs of microspheres formulation (TBHMs V) are shown in **Fig. 3**. Non-aggregated microspheres with spherical shape were obtained. Moreover, formulation (TBHMs V) showed a smooth surface, indicating that TBH might be well dispersed inside the carrier. The sphericity factor was obtained in the range of 1.01 to 1.05, indicating that the prepared formulation (TBHMs V) has spherical particles.

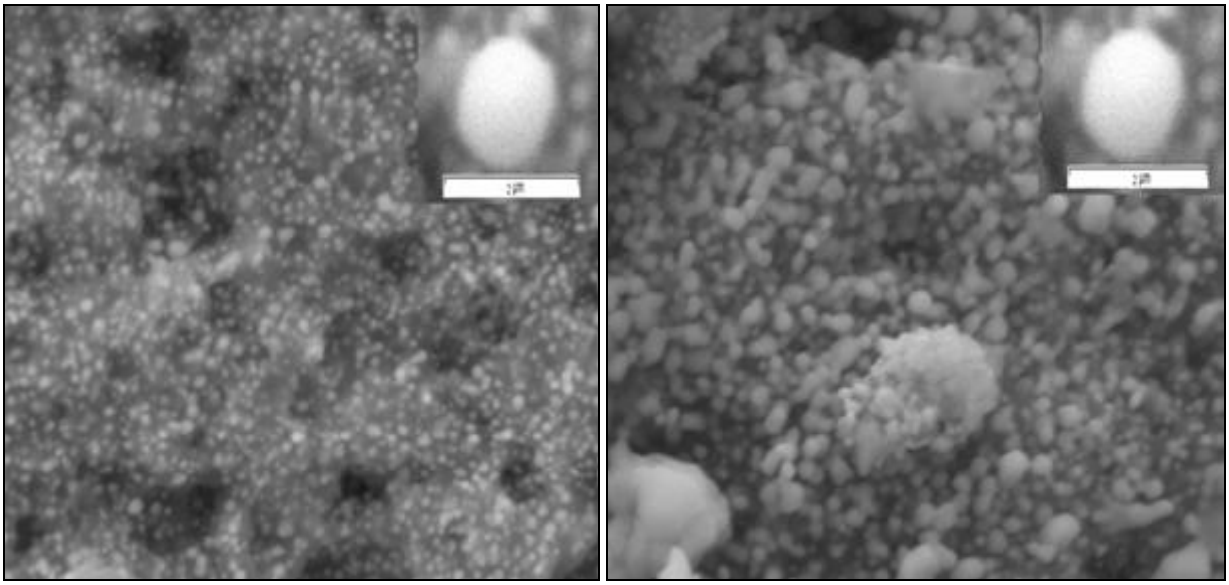

FIG. 3: SEM PICTURES OF MICROSPHERE FORMULATION (TBHMS V) AT: A) 1500× B) 2400×

Drug Loading and Encapsulation Efficiency: Encapsulation efficiency ranged from 58 to 89 %. Generally the encapsulation efficiency and drug content increases with increasing amounts of polymers in the microspheres. Formulation (TBHMs V) showed relatively higher encapsulation efficiency indicating high polymer concentration. It can be inferred from the results that there was a proper distribution of TBH in the microspheres. During the microencapsulation process, mechanical variables cause loss of the final product and hence process yield may not be 100%. Formulation (TBHMs V) showed maximum drug loading of 28.1 - 29.6 %, respectively. The results obtained are given in **Table 1**.

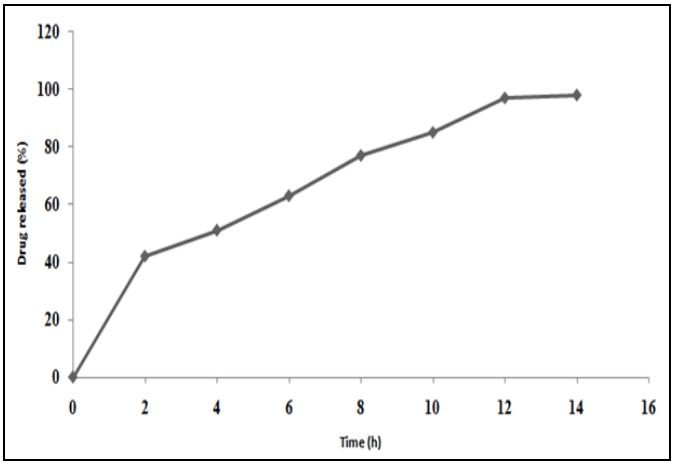

FIG. 4: DRUG RELEASE PROFILE OF TERBINAFINE FROM MICROSPHERES (TBHMS V) (MEAN ± SD, n=3)

***In vitro* Drug Release Studies:** The release profile of the drug from microspheres clearly indicates that the concentration of polymers slows the release of TBH from microspheres. At the end of 12 hr, *in vitro* drug release from formulation (TBHMs V) was found to be 98.8 % in the vaginal environment, as shown in **Fig. 4**. The total cumulative quantity of the drug released at the end of the 12 hr dissolution test was below 100%. This may be in part due to the relatively slow erosion of the matrix under these test conditions, with a resultant slow release of entrapped drug from the matrices undergoing testing.

***In vitro* Antifungal Studies:** An antifungal study with Sabouraud Dextrose Agar medium showed that the TBH loaded with microspheres was able to control (inhibit) the growth of *Candida albicans* for more than 24 hr. The formulation (TBHMs V) showed an average zone of inhibition of 19.3 ± 0.5 mm, which was higher compared to the average zone of inhibition of pure Terbinafine (TBH) *i.e.* 14.4 ± 0.4 mm. Also there was no significant effect produced by placebo polymer which implies that the polymer as such has no activity and no interference with the activity of the drug.

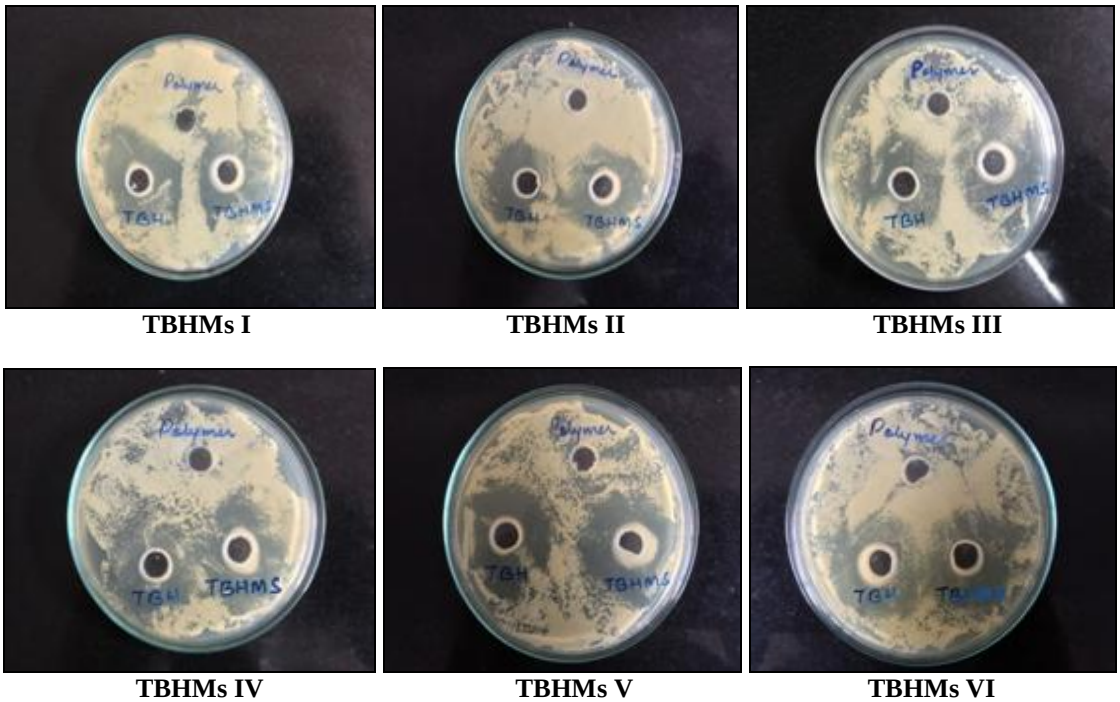

FIG. 5: *IN VITRO* ANTI FUNGAL ACTIVITY OF TERBINAFINE LOADED MICROSPHERES (TBHMS V) IN COMPARISON TO PURE DRUG (TBH) USING *CANDIDA ALBICANS* AFTER 24 HRS

CONCLUSION: The system of TBH loaded microspheres for mucosal vaginal drug delivery has demonstrated their effectiveness for the intended action. The *in vitro* anti fungal activity and drug release studies have shown the ability of the microspheres formulation (TBHMs V) to adhere to the vaginal mucosa for an extended period of time as well as to improve drug availability. It can be concluded from the results of the present experimental work, that the microspheres formulation (TBHMs V) is easy to administer, simple and comfortable. Hence, terbinafine could be formulated into this type of drug delivery system for controlled drug release.

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CONFLICTS OF INTEREST: Nil.

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