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# FORMULATION, EVALUATION AND MICROBIAL ASSAY OF TINIDAZOLE VAGINAL SUPPOSITORY CONTAINING *LACTIC ACID BACILLUS SPORES*

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**Abstract:** Human vagina represents a dynamic ecosystem dominated by certain species of *Lactobacillus*. This microorganism restricts the growth of pathogens by using properties of steric exclusion and inhibitory substance production. Serious complications including bacterial vaginosis and vaginal cancer are often determined in women with reduced numbers of lactobacilli. Local application of *Lactobacillus* is consequently promising to keep the vagina colonized by this strain, which consequently reduces the infections. The first objective of this research was to develop a local application pharmaceutical formulation of a vaginal suppository containing lyophilized culture of *Lactobacillus* with anti-microbial agent tinidazole. The second objective was to establish its in vivo performance by developing in vitro methods of evaluation as well as antimicrobial activity of tinidazole alone with *Lactic acid Bacillus Spores* in vaginosis.

**Key Words:** Vaginosis, *Lactobacillus Spores*, Tinidazole, Formulation, Evaluation.

## INTRODUCTION

The present research and study is directed to Anti-microbial and lactic acid bacillus combination in a comprising pharmaceutical acceptable carrier and the methods for treating fungal, bacterial, protozoal and yeast infection. Some of the most common pathogens associates with invasive fungal infections are the opportunistic yeast, such as *Candida spp*. and *Asppergillus spp*. thousands of *Candida spp* cells can be present in an individual, primarily in the gastrointestinal tract, as a harmless commensal organism. However, *Candida spp*., such as *C. albicans*, causes opportunistic fungal infections. Infections can be localized such as a vaginal infection or an oral infection, both of which cause a considerable degree of discomfort. The objective of this study was to develop a vaginal suppository containing *lacti acid bacillus spores*. Further the present research study provided the combination of anti-infective drug tinidazole with microorganism lactic acid bacillus spores in a pharmaceutical formulation as suppository. [1-3]

### *Bacterial vaginosis (BV)*

BV is a clinical syndrome associated with a group of pathogenic microorganisms rather than specific pathogen. It is a very common manifestation amongst the women population. Though the exact causative pathogen has not been figured out, it has been observed that there is a corresponding decrease in the population of the lactobacilli species. This results in the increase in the pH of the vaginal lumen due to the reduction in the lactic acid production. Apart from the lactic acid, the production of lactocin and H2O2 also receives a setback. In general, the *lactobacilli* are replaced with the increased population of pathogenic gram negative anaerobic bacteria like *E. coli, G. vaginalis, M. hominis* and *M. Curtisii.* Bacterial vaginosis (BV) is characterized by an alteration of normal vaginal microflora in which a mixed anaerobic bacterial flora becomes prevalent over the population of lactobacilli. The common organisms causing a vaginosis as *Gardnerella vaginalis, Candida albicans. candidiasis, genital candidiasis,* or *vulvovaginal candidiasis, Trichomonas vaginalis, Chlamydia trachomatis, Neisseria gonorrhoeae, the herpes simplex virus, the human papilloma virus (HPV), Gardnerella vaginalis, Mobiluncus, Bacteroides* and *Mycoplasma*.[1-6]

### *Lacto bacillus spores*

*Lactobacillus* refers to a group of lactic acid producing bacteria that make up many of the 400 normal probiotic species in the human body. Lactobacilli are "friendly" bacteria, meaning that they normally occur in the human gastrointestinal and genitourinary tracts and play important roles in promoting good health. The presence and dominance of *Lactobacillus* in the vagina is associated with a reduced risk of bacterial vaginosis and urinary tract infections. The mechanisms appear to involve anti-adhesion factors, by-products such as hydrogen peroxide and bacteriocins lethal to pathogens. In the present study, *lactic acid bacillus spores* since it gives better releasing rate in a conventional suppository of Water Soluble/Water Miscible Bases polyethylene ethylene: carbopol base [7-20].

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***Tinidazole***

Tinidazole demonstrates activity both in vitro and in clinical infections against the following protozoa: Trichomonas vaginalis, Giardia duodenalis (also termed *G. lamblia*), and *Entamoeba histolytica*. Tinidazole does not appear to have activity against most strains of vaginal lactobacilli. It has been used for trichomoniasis, amoebiasis and giardiasis. Tinidazole is active against a wide range of bacteria including Bacteroides spp. Anaerobic cocci, fuso bacterium spp. Clostrium spp. and gardnerella, Vaginalis. It is also effective against protozoa including Trichomonas spp.Entamoeba histolytica and Glardialamblia.

***Mechanism of Action***

Tinidazole is a prodrug and antiprotozoal agent. The nitro group of tinidazole is reduced in Trichomonas by a ferredoxin-mediated electron transport system. The free nitro radical generated as a result of this reduction is believed to be responsible for the antiprotozoal activity. It is suggested that the toxic free radicals covalently bind to DNA, causing DNA damage and leading to cell death. [21-26]

## MATERIAL AND METHOD

Tinidazole I.P was a gift sample from Alpa Laboratory Ltd., Indore, Madhya Pradesh. Poly Ethylene Glycol 6000-8000 and carbopol 934 purchased from Central Drug House (P) Ltd., New Delhi. *Lacto bacillus spores* also were gifted from Sanzyme Ltd Banjara hill, Hyderabad. All other chemicals and reagents were used of analytical grade.

***Preparation of Suppositories***

The 20 vaginal suppository were prepared with the same combination as *lactic acid bacillus spores*. Tinidazole and bases Polyethelen glycol (PEG 6000-8000), Carbapol 934 (1%) as shown in table 1.

The conventional suppositories were prepared by fusion method. The Carbapol 934 (1%) was used as a muco-adhesive agent and PEG (6000-8000) as the suppository base which was melted over the water bath, then carbapol 934, followed by drug was added to the melted base with continuous stirring. Finally, lyophilized *Lactobacillus Spore* was added in the melted base at the temperature about 40-45°C with gentle stirring until a homogeneous mass was produced. After that the mixture was poured into a metal suppository mold at a temperature just above the congealing point of the suppository base and cooled over the ice bath. The mold was then allowed to solidify for 1 hour at room temperature and finally all the prepared suppositories were kept in the refrigerator for further studies. [27, 28]

**Table 1:** Formulation of Tinidazole Suppository

| S.No | Ingredients | Qty taken in gms | Actual qty to be taken for 1 suppository |
|---|---|---|---|
| 1. | Tinidazole I.P | 0.2gm | 200 mg |
| 2. | *Lactobacillus Spore* 150 million | 1 gm | 1000 mg |
| 3. | Carbapol 934 | 1% | 50 mg |
| 4. | Poly Ethylene Glycol 6000-8000 | q.s | q.s |
| | Total | 5 gm | 5000 mg |

**Evaluation of Vaginal Suppositories:**

***Physical evaluation***

***Test of appearance: odour, colour, shape, surface condition:*** Colour and the surface characteristics of the suppositories are relatively easy to assess. It is important to check for the absence of fissuring, pitting, fat blooming, exudation, sedimentation, and the migration of the active ingredients. [30-32] Suppositories can be observed as an intact unit and also by splitting them longitudinally, the result shown in table 2.

**Table 2:** Test of Appearance, Odour, Colour, Shape, Surface Condition

| Sr. No. | Physical characteristics | Formulation Tinidazole suppository |
|---|---|---|
| 1 | Shape | Ogive |
| 2 | Surface condition | Smooth |
| 3 | Color | Half white |
| 4 | Odor | Odourless |

***Weight Variation Test***

The weight variation test was determined according to the British Pharmacopoeia. Twenty suppositories were weighed individually and the average weights were determined. No suppositories should deviate from average weight by more than 5% except two, which may deviate by not more than 7.5%, shown in table 3. [32]

**Table 3:** Physico-Chemical Characterization formulations.

| Parameters | Formulation |
|---|---|
| Weight variation * | 5.0307 ± 0.1528 |
| Hardness * | 1.90 ± 0.12 |
| Melting time* | 37.6 - 41.6 ± 0.51 |
| Softening time* | 10.24 ± 0.04 |
| Disintegration time* | 13.34 ± 0.14 |
| Content uniformity* | 98 % ± 1.53 |

* All Values Represents Mean + sd, n=6

***Test of physical strength:***

***Hardness (breaking) test:*** The hardness of 10 suppositories from each batch was determined by cutting the middle portion of suppository. It was measured in its diametric direction using Monsanto hardness tester. Result shown in table 3. [32]

***Test for melting range:*** The ascending melting point method was used to determine the melting point of each type of suppositories. Capillary tubes, 10 cm in length, sealed at one end, were filled with the formulation to about 1cm height, and then it was dipped in gradually heated electro-thermal thermometer. The melting temperature was recorded when the suppositories started to melt.
Results shown in table 3 [33-34]

**Test of softening time:** It measures the time necessary for suppository to liquefy under pressure similar to those bound in rectum/vagina in presence of water at body temperature. [32-35] shown in table 3.

***Disintegration Test (tablet disintegrator):*** Randomly six suppositories were selected from each batch for disintegration test. Disintegration test was performed without disc in citric acid/phosphate buffer solution pH 4.4 maintained at 37 ± 0.5°C using USP disintegration apparatus (Electrolab ED-2L). The suppository to be tested was placed in a cylindrical glass container with perforated ends and immersed in 1,000 ml of citric acid/phosphate buffer solution pH 4.4 maintained at 37 ± 0.5°C. The cylindrical glass container was moved up and down in the buffer. The time for disintegration was noted, which should not more than 60 minutes as per BP. shown in table 3. [30-32]

***Chemical Evaluation:***

***Test for uniformity:*** Three suppositories were randomly selected from each base and assayed individually for drug content. The suppository was melted with gentle heating in a water bath in the presence of 25 mL of phosphate buffer solution, pH 7.4. The volume was adjusted to 250mL with phosphate buffer. The flask was agitated on a shaking water bath at 37°C for 4 h. After centrifugation and filtration, the UV absorbance of the solution was measured spectrophotometrically at λmax 314 nm against a blank solution prepared by treating plain suppositories in the same manner [29] shown in table 3.

***Release Studies/diffusion/dissolution rate:***

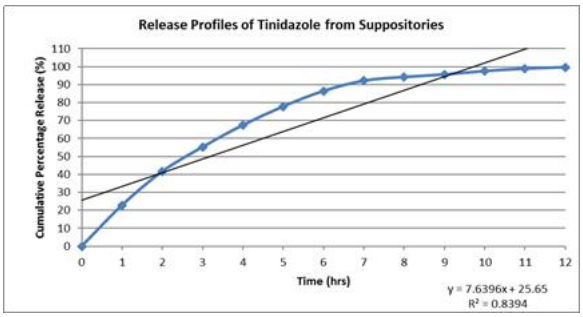


**Fig 1:** Release profile of Tinidazole from suppository

***In vitro release kinetics of tinidazole Suppository:*** In vitro release study was performed by using USP type1 rotating basket apparatus (Electrolab TDP-06P). Dissolution medium was 500 ml citric acid/phosphate buffer solution pH 4.4 (modeling the vaginal pH) was used as the medium. Rotation speed was controlled at 120 rpm while temperature was maintained at 37±0.5°C. The suppository was inserted into the basket using stainless steel forceps. Five milliliter aliquots of the dissolution fluid were withdrawn at specified interval from the reservoir and each time replaced with equal volume of fresh dissolution medium. Withdrawn samples were suitably processed & diluted and analyzed using double beam UV/Vis spectrophotometer at 314 nm for tinidazole suppository. [29]

***Viability Test and Stability of spores:*** The vaginal suppositories containing Lactobacillus Sporogenes were kept in glass containers at ambient temperature (30±2°C) and 2-8°C for 3 months. At appropriate time intervals, 0, 1 week, 2 week, 3 week and 4 week, the survival of lactobacillus was determined by plate method using MRS agar medium result shown in table 4. [20-21, 27]

**Table 4:** Viability of *Lactobacillus Sporogenes* from Tinidazole Suppositories

| Time Period | CFU (Colony Forming Unit) Ambient temperature | | | 2-8°C (Cool Storage) | | |
|---|---|---|---|---|---|---|
| 0 Day | $5.72 \times 10^5$ | $5.61 \times 10^5$ | $5.84 \times 10^5$ | $5.72 \times 10^5$ | $5.61 \times 10^5$ | $5.84 \times 10^5$ |
| 1st week | $3.12 \times 10^5$ | $4.87 \times 10^5$ | $5.23 \times 10^5$ | $4.31 \times 10^5$ | $4.42 \times 10^5$ | $4.41 \times 10^5$ |
| 2nd week | $4.13 \times 10^4$ | $3.97 \times 10^4$ | $4.21 \times 10^4$ | $4.11 \times 10^5$ | $4.04 \times 10^5$ | $4.18 \times 10^5$ |
| 3rd week | $1.61 \times 10^4$ | $1.92 \times 10^4$ | $1.81 \times 10^4$ | $3.67 \times 10^5$ | $3.52 \times 10^5$ | $3.81 \times 10^5$ |
| 4th Week | $3.91 \times 10^3$ | $3.82 \times 10^3$ | $4.05 \times 10^3$ | $3.18 \times 10^5$ | $3.21 \times 10^5$ | a. $10^5$ |

***Microbial Evaluation / Assay:***

A standardized inoculum of bacteria/fungus is swabbed onto the surface of a Mueller Hinton Agar/ Sabouraud Dextrose Agar plates. Sample of antimicrobial agents are loaded in well in the agar.

***Table 5:*** *Microbial Strains*

| S.No | Strain | Organism | Media |
|---|---|---|---|
| 1 | *Staphylococcus aureus* ATCC 25923 | Gram Positive | Mueller Hinton Agar |
| 2 | *Bacillus subtilis* ATCC 13597 | Gram Positive | Mueller Hinton Agar |
| 5 | *Aspergillus niger* ATCC 9029 | Fungal Strain | Sabouraud Dextrose Agar |
| 6 | *Candida albicans* ATCC 24433 | Fungal Strain | Sabouraud Dextrose Agar |

**Table 6:** Summary of Results of Antimicrobial Activity

| S.No | Formulations | MIC Concentration (µg/mL) | | | |
|---|---|---|---|---|---|
| | | *Staphylococcus aureus* | *Bacillus subtilis* | *Aspergillus niger* | *Candida albicans* |
| 1 | Tinidazole suppositories | 16 | 8 | >64 | >64 |

After overnight incubation, the diameter of zone of inhibition is measured around each disk, and the experiment was carried out with help of Indian Pharmacopeia, NCLS Guidelines [27-28]. Stains used, shown in table 5 and observation in table 6 and 7.

**Table 7:** Antimicrobial Activity Of Tinidazole Suppositories

| S.No | Dose of Formulations (µg/mL) | Zone of inhibition in diameter (mm) | | | |
|---|---|---|---|---|---|
| | | *Staphylococcus aureus* | *Bacillus subtilis* | *Aspergillus niger* | *Candida albicans* |
| 1 | 64 | 14 | 16 | 4 | 3 |
| 2 | 32 | 15 | 16 | 0 | 2 |
| 3 | 16 | **12** | 15 | 0 | 0 |
| 4 | 8 | 7 | **12** | 0 | 0 |
| 5 | 4 | 8 | 7 | 0 | 0 |
| 6 | 2 | 8 | 8 | 0 | 0 |
| 7 | 1 | 6 | 8 | 0 | 0 |
| 8 | 0.5 | 0 | 6 | 0 | 0 |
| 9 | 0.25 | 0 | 6 | 0 | 0 |

***Stability Studies:***

Suppositories were wrapped in the aluminium foil and kept in stressed condition by six cycles of freeze (2-8°C) and thaw (25°C) process. Suppositories were also kept in accelerated condition temperature (30°C) for 45 days [28]. Suppositories were examined visually and drug content as per the procedure of content uniformity, result shown in table 7.

**Table 8:** Stability Study of Tinidazole Suppository

| S.No | Days | Freeze and Thaw (Six Cycles) | | Accelerated Temperature | |
|---|---|---|---|---|---|
| | | Physical Changes | % drug Content ± S.D. | Physical Changes | % drug Content ± S.D. |
| 1 | 0 | No significant changes were Seen | 98.70 ± 0.55 | No significant changes were Seen | 98.26 ± 0.10 |
| 2 | 15 | No significant changes were Seen | 97.64 ± 0.42 | No significant changes were Seen | 96.77 ± 0.62 |
| 3 | 30 | No significant changes were Seen | 96.28 ± 0.88 | No significant changes were Seen | 93.78 ± 1.30 |
| 4 | 45 | No significant changes were Seen | 94.95 ± 1.57 | No significant changes were Seen | 91.37 ± 1.06 |

## RESULT AND DISCUSSION

In the current study, successful attempts were made to develop lactic acid spore containing tinidazole suppositories for the treatment of vaginosis. The formulations were tested under *in vitro* conditions on fungal and bacterial culture taken as a model causative organism, microbial assay, viability test as well as evaluated for the physicochemical parameters such as appearance, physical properties, drug content, *in-vitro* dissolution and stability studies. All the physical characteristics of 20 suppository formulation shown in table 2, appearance was to give shape, odourless with smooth surface and half white colour because of PEG 6000-8000: carbapol base used. The other physico-chemical characterization of the formulations as shown in table 3, weight variation of all suppositories were within the acceptable limit of 100% ± 5%. The breaking strength of all suppositories shown in table 3, were between 1.2-2.0kg/cm which was good for the expected results. The average melting range 37.6 - 41.6°C shown in table 3, which was satisfactorily to melt at normal body temp 37° C. Product's liquefaction time was measured and the average softening time was 10.24 ± 0.04 minutes which is up to the mark, shown in table 3, since in general liquefaction should take no longer than about 30 minutes. The mean disintegration time as shown in table 3, was 13.34 ± 0.14 minutes which was matching with acceptable limits, according to BP the disintegration time of each suppository should be less than 60 minutes. The drug content of all the suppositories was determined spectrophotometrically at 310 nm shown in table 3. It varied from 98.22 to 99.41 % which was at acceptable limit of 85% -115% of the label claim for suppository.

*In-vitro* dissolution/release rate profile were determined by spectrophotometrically at 310 nm shown in Fig.1, the Cumulative Percentage release was noted for 12hr as Carbapol 934 , one of the base of formulation giving some sustain released action of a drug which was found to be 99.64% at acceptable limit.

Viability Test and Stability of spores, sufficient growth of the *Lactobacillus* ($10^5$ colony-forming units/ml) on 0,1,2,3,4 weeks at ambient and 2-8° C temperature respectively, was observed when grown on a standard MRS medium plate as shown in the table 4. Colony characteristics and gram staining confirmed the presence of *Lactobacillus*. This indicates that the viability of the *Lactobacillus* was not affected during preparation of the formulation. Microbial test and stability studies were found to be well within the limits and official standards.

Microbial Evaluation/Assay as shown in table 5 and 7, was determined by minimum inhibitory concentration (µg/mL) and by measuring the zone of inhibition (diameter in mm), the formulation was found to be the effective against the *Staphylococcus aureus, Bacillus subtilis, Aspergillus niger, Candida albicans,* microorganisms which are the primary causative organism for bacterial vaginosis.

Stability studies of suppositories were examined on the day 0,15,30,45 at freeze and at accelerated temperature for percent drug content and physical changes, shown in table 8. It was noted that there were no significant changes in physical and percent drug content seen in the formulation unit respectively.

## CONCLUSION

It was concluded that the bioactive dosage formulation containing anti-microbial agent with *L. sporogenes* appears to be a good candidate for probiotic prophylaxis and treatment of vaginal infections. The developed assembly was satisfactory in simulating the application site. The viability of *L. sporogenes* was not affected during preparation of the suppository and the inhibitory action of the tinidazole was satisfactorily. Thus, the suppository formulation containing *Lactobacillus* and the methods of its evaluation developed in this research work may be beneficial in preventing bacterial vaginosis. Further investigations have to be carried out in antimicrobial activity with lacto bacillus spore in the bacterial viginosis treatment is needed.

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