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PREPARATION AND EVALUATION OF PLANT BIOACTIVE PHYTOCHEMICAL BASED BIOADHESIVE ANTIFUNGAL VAGINAL FORMULATION

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Abstract:
The present experimental study was designed with an aim to develop an effective bioadhesive antifungal vaginal gel using suitable combination of plant bioactives like curcumin (CUR) in combination with itraconazole (ITR). The combination curcumin of and itraconazole were optimized based on in-vitro antifungal study. Different Bioadhesive polymers like carbopol P934, carbopol 940 and HPMC K4M either individually or in suitable combination were used to fabricate Bioadhesive gels. Essential in-vitro studies such as screening of antifungal activity, rheological property, spreadibility, pH, Content uniformity, capacity of mucoadhesion, drug release etc. were performed to evaluate the performance of prepared gel with respect to its safety and efficacy. Results of the study reveal a significant increase in antifungal activity of itraconazole in presence of curcumin. Againas per as performance of dosage form concern formulations prepared using carbopol found suitable for vaginal drug delivery.

Key Words: *Bioadhesive vaginal gel, Itraconazole, Curcumin, Synergestic, Antifungal*

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INTRODUCTION:

The available reports and literature describes that, from the early beginning infectious diseases caused by pathogenic bacteria and fungi are remained most devastative life threatening issue for the society. Vaginal candidacies are a common fungal infection mostly caused by Candida species, especially *Candida albicans*, and suspected to be the second most predominant mucosal infection after bacterialvaginosis[1]. The major symptoms of vaginal candidiasis include dyspareunia, pruritis, burning sensation, itching, dryness, soreness and some time may associate with white discharge along with pain [2].Approximately 75% women population experience vaginal candidiasis during their life and about 40% to 50% of them suffer multiple episodes. [3,4]. It is highly essential to understand the pathophysiology of the disease before to develop safe and effective remedies. Candida albicans is not a pathogen, but when local or systemic defense mechanism of the host get afflicted, it became viable. Under normal healthy conditions, lactobacillus present in vaginaproduces lactic acid, which act as buffer and maintains the pHof vagina in the range 4–5 (acidic), againpresence of hydrogen peroxide (H_2O_2) and bacteriocins in vaginal fluid resist the overgrowth ofpathogenic microbes. Under certain ill conditions, when the local or systemic defence mechanism compromised, thisbalance gets disturbed and as result, excessive overgrowth ofCandida sp. and depletion Lactobacillus spp. takes place, thatfinally leads to damage to vaginal epithelium and then symptoms get triggered and Vaginal candidacies occurs [5].However, this kind of infections can be managedeasily, following proper antifungal therapy. However its became critical issue due to several reasons like, lake of proper awareness, patients hesitation due to social issue that resultsuntreated clinical condition,immunological state of the patient, restricted number of commercially available effective antifungal drugs, Delay in diagnosis of the infection, chances of resistance develop on the therapeutic agents [6]. Therefore, became challenging clinical issue, which needs to be addressed on priority basis.

Hemaiswarya S. *et al.*suggested that, vaginal infections due to Candida Spp. Can effective management using both systemic and topical antifungal therapies [7].Again, Choudhury A.*et al.* also suggested that application of topical formulation not only helps to maintain the pH of vaginal surrounding but also it minimise other clinical symptoms like burning sensation, itching and dryness, which may be advantageous for patents [8].Further, limitation associated with systemic antifungal therapy like short-term treatment, avoidance of fast-pass metabolism, higher concentration of drug at the targeted site and other side effects, makes topical antifungal therapy a rational choice for treatment of vaginal candidiasis [9].

Itraconazole (ITR) is a new triazole antifungal drug with abroad spectrum of activity, considered as an attractive alternative for treatment of vaginal candidiasisbecause of its reported effective activity against Candida species [10]. However, unlike fluconazole, it also shows serious sensitivity issue, when applied topically at high concentration [11]. Therefore looking towards the safety as well as efficacy aspect, there is a need of an alternative approach for the topical delivery of ITR. Further, looking towards the recent research in the field of combination drug therapy, it might consider as suitable approach in this regards.

Plant-derived bioactive phytochemicals are always remains asa source ofnovel therapeutics. Recent research on this field indicates when bioactive phytochemicals are applied in combination with existing drug shows remarkable increase in efficacy. Again in several instance are which states that, bioactive phytochemical may synergistically improve the overall effect of combination [12]. Bioactive phytochemicals like curcumin is reported as attractive prototypes for this purpose due to their broad spectrum of biological activities.curcumin, a natural compound found in the *Curcuma longa* plant, active against different bacteria, fungi and parasites, made it a good candidate to enhance the inhibitory effect of existing antimicrobial agents through synergism [13,14]. Curcumin have the capacity to prevent the adhesion of Candida species to the host epithelial cell, and reported to have significant inhibitory effect against fungi due to its membrane-lytic capacity [13,15]. On the other hand, itraconazole (ITR) mainly works based on the mechanism of target heme protein, cytochrome P450. [10,16] Therefore it is expected that, combination of curcumin and itraconazole may show increase therapeutic activity against *Candida albican* infection.

Therefore, this research work has been designed with a primary objective to develop a safe and effective antifungal bioadhesive vaginal gel formulation for the management of wide range of fungal infections. An antifungal screening study has been carried out to estimate efficiency of combination against *Candia albican*. Again bioadhesive gels were prepared incorporating optimized drug combination using different ratios of polymers like HPMC, CarbopolP934 and Carbopol 940. All the prepared

formulations were submitted for different In-vivo and In–vitro evaluation.

MATERIAL AND METHODS:

Materials:

The pathogenic antifungal stain of *Candida albicans* (MTCC 227) was purchased from MTCC Chandigarh, Materials used for the experimental work such as; itraconazole was obtained as gift sample from Ipca laboratories India. Carbopol 940, Carbopol P 934 and HPMC were purchased from S.D. fine Pvt. ltd., RPMI 1640 media was and 96 well plates purchased from sigma, Triethanolamine & Glycerin was purchased from loba Chemie Ltd. India.

Methods:-

Determination of minimum inhibitory concentration (MIC) &Fractional inhibitory concentration index (FICI)

The MIC value of each APIs was evaluated using broth dilution methods as per standard guideline of NCCLS, M27. At first the *Candida albicans (*MTCC 227*)* strain was subculture in Sabouraud dextrose agar media to ensure purity and viability. After that a standard pathogenic cell suspension was prepared by suspending few colonies from a freshly prepared culture, in 5 ml of saline solution. Final inoculums of 4 x 10^6 cells per mL were prepared by vortexes the suspension for 30 sec followed by adjustment the transmittance as per McFarland standard. After that a standard sterile stock solution of itraconazole and curcumin, individually as well as in suitable combination were prepared. MIC value of individual drug and drugs combination was measured on difference in optical density through 96 plate method [29].

The effect of combination of itraconazole and curcumin was investigated based checkerboard experiments [8,28]. A 100µl aliquot of working cell suspension was placed into 96-well microtitre plate containing RPMI 1640 medium. Again different concentration of itraconazole and curcumin, alone as well as in combination were placed vertically and horizontally into the plates. Potentiality of combination was measured after proper incubation for 48 hours, by means of the fractional inhibitory concentration index (FICI) value using the following equation [6,7, 28].

FICI = FIC of curcumin + FIC of Itraconazole

Where,

FIC of curcumin = MIC of curcumin in combination with FLC/ MIC of curcumin alone,

FIC of itraconazole = MIC of itraconazole in combination with CUR / MIC of itraconazole alone.

FICI values= 0.5, represent synergistic interactions, 4.0 antagonistic effect and values in between these two represent no interaction

Preparation of Bioadhesive Gel

Bioadhesive gels were prepared using different gel forming polymers namely Carbopal P 943, Carbopol 940, Hydroxy-propyl-methyl cellulose either individual or in combination. Accurately weighted required quantities of polymers as well as selected antifungal combination were transferred to beaker containing desire quantity of hydro-alcoholic (methanol & water) solvent system. Whole content were stirred for 5-10 min by means of magnetic stirrer and allowed to hydrate for 12 hours. After that a few drops of triethanolamine as neutralizing agent, glycerin as a moistening agent along with propylene glycol were added to the hydrated mass and mixed slowly with continuous gentle stirring by means of magnetic starrier until the homogenous gel was formed [8,17].

Table 1: Formulation design of Bioadhesive vaginal gels

S. N	Materials	F1	F2	F3	F4	F5	F6	F7	F8	F9
1	Carbopol P 934	1 %	-	-	0.5 %	1 %	1.5 %	-	-	-
3	HPMC	-	1%	-	1.5 %	1 %	0.5 %	1.5%	1%	0.5%
2	Carbopol 940	-	-	1%	-	-	-	0.5%	1%	1.5%
4	Water	90ml	90ml	90ml	90ml	90ml	90ml	90ml	90ml	90ml
5	Itraconazole	0.625 mg	0.625 mg	0.625 mg	0.625 mg	0.625 mg	0.625 mg	0.625 mg	0.625 mg	0.625 mg
6	Curcumin	0.175 mg	0.175 mg	0.175 mg	0.175 mg	0.175 mg	0.175 mg	0.175 mg	0.175 mg	0.175 mg
7	Methanol	5 ml	5 ml	5 ml	5 ml	5 ml	5 ml	5 ml	5 ml	5 ml
8	Glycerin	5 ml	5 ml	5 ml	5 ml	5 ml	5 ml	5 ml	5 ml	5 ml
9	Triethanalamine	0.18ml	0.18ml	0.18ml	0.18ml	0.18ml	0.18ml	0.18ml	0.18ml	0.18ml

Evaluation of Prepared Bioadhesive Gel

Visual and Organoleptic Examination

The prepared gel formulations were visually inspected for their color and appearance. [8,17] Prepared formulations were seems to be homogeneous, free from any gritty particles and slightly yellowish in color

Compatibility Study.

In this study physical mixture of individual drugs and all incorporated polymers in single as well as in combination were analyzed by means of FTIR study (Mekkawy A. *et al.,* 2013). The major peaks found in physical mixture of drug with polymer are compared with the peak of individual APIs.

Spreadability study:

The study was performed using modified parallel plate method to determine the spreadability [8,17,19]. The prepared formulations were placed in between a set of 20×20 cm glass slides & around 100 g weights were placed upon the upper slide, so that applied gel can spread uniformly. Then the weight was removed and scrapped off the excess of gel adhering to the slide. The set of slides were fixed in such a way that only upper slide may slip off freely due to the weight tied with it. The time taken for the upper slide to separate from the lower slide was noted. The experiment was carried out three times and the average of three reading was recorded. Following formula was used for calculation-

S = M.L/T[Where, M = weight tied to upper slide; L = Length of glass slide; T = Time taken to separate the slide]

Percentage Yield:

In this study weight of empty container as well as of gel formulation along with container was measured respectively. Then difference between the weight empty container and weight of container with gel formulation were measured, that considered as practical yield where as the total weight of each ingredient used in each formulation was considered as theoretical weight [8,20,23] The percentage yield was calculated using the formula as below-

$$\textbf{Percentage yield} = \frac{\text{Practical yield}}{\text{Theoretical yield}} \times 100$$

Determination of Drug Content

Around 10 gm of prepared gels were transferred into a 100ml volumetric flask containing 50ml of phosphate buffer pH 4.5., under continuous agitation for 5hr by means of mechanical rotary shaker. Further the mixture was kept aside for 24hrs in order to get complete release of drug from gel base. After that the content was filtered using Millipore filter (0.45µm) and absorbance was measured After suitable dilution using UV- visible spectrophotometer (UV – 1700, Shimadzu, Japan) at λ_{max} 262 nm and 422 nm respectively using buffer (pH 4.5) as blank [3, 8, 17].

Determination of pH :

The pH of gels was determined using a digital Electronic pH meter. Initially the pH meter was calibrated using standard buffers of pH 4, 7 and 9. Accurately 5 gm of gel was weighed and dispersed in 50 ml of double distilled water. The electrode of pH meter was dipped in dispersion and the numerical value displayed in pH meter was noted [17, 20, 21, 23].

Viscosity and Rheological Studies

The viscosity of prepared gels was determined with the help of Brookfield viscometer [8,22]Formulations were placed in the sample holder and suitable spindle attached perpendicularly inside the sample. The spindle was attached to viscometer and allowed to rotate at a constant speed. The reading displayed on viscometer was measured.

Bioadhesion study:

Bioadhesion study was performed using Pig vaginal mucosa as a model. Skins of vaginal area were obtained from a local slaughter house. Vaginal mucosa was carefully separated from underlying tissues and treated with normal saline. There after it was cut into smaller pieces of adequate size. After that a single part of mucosal tissue was attached perfectly to the back side of owing balance such a way that it remain tightly attached till the completion of study. To complete the study a glass slide was taken and required amount of formulated gels were spread over it in such a manner that may cover the whole area of mucosal tissue when come in contact together. The slide and the tissue attached in the pan were fixed for 5min. On the other hand of the pan a weight of 5gm was applied and determined the time taken by the tissue to detach from the glass slide were measured [8,22,24].

***In-Vitro* Drug Release Study**

The apparatus consists of a glass cylinder with both the ends open, 10 cm in height, 3.8 cm in outer diameter and 3.2 cm in inner diameter was used as a permeation cell. A cellophane membrane previously soaked in distilled water for 24 hours was fixed to the one end of the cylinder. 10 mg of gel was taken in the cell (donor compartment) and the cell was immersed in a beaker containing 100 ml of buffer of pH 4.8 (receptor compartment). The whole assembly was fixed in such a way that the lower end of the cell containing gel was just touched (1-2 mm deep) to the diffusion medium, the medium in the compartment

was agitated using a magnetic stirrer at the temperature 37±1ºC [8,22, 25]. Sink condition were maintain throughout the experiment and after suitable dilution; the sample was analyzed by using Shimadzu UV visible spectrophotometer at 262nm and 422 nm respectively.

Vaginal irritation test

The primary vaginal irritation test was performed on Newzealand white female rabbit (1.5-2.5kg). All the animals were kept under standard laboratory condition. The total numbers of animals were divided into four batches, each batch containing three animals. 1ml of prepared gel was inserted daily, for 10 days, through a lubricated catheter into the vagina of rabbits [26,27]. The external genitalia are observed regularly for any signs of oedema, erythema or discharge as a reaction to the exposure to the test materials. The experimental protocol of the study was approved by the Institutional Animal Ethics Committee (Regd. No. CIP / IAEC / 2013-14/044).

RESULT AND DISCUSSION:

The present investigation was carried out to determine capacity of combination against fungal infections cause due to *Candida albicans* and to estimate the performance and suitability of the prepared bioadhesive vaginal gel. In this context in-vitro antifungal activity of itraconazole and curcumin alone as well as in combination were tested against Candida albicans. The MIC value of itraconazole and currcumin alone was found 64ug/ml and 128ug/ml. however; a remarkable response was observer when used in suitable combination. To explore the finding, the study was further extended to determine the mechanism involved behind such effect. The study of fractional inhibitory concentration index shows that, when the curcumin and itraconazole added in suitable concentration results synergistic action as mentioned in (table no-03), which improves efficacy of itraconazole against *Candida albicans*, that indicates, combination of curcumin and itraconazole in suitable concentration ratio may use effectively for the management of such type of fungal infections.

Before preparation of dosage forms, the compatibility of drugs and excipients were determined using FTIR study. The results indicate no such interaction observed.(fig-03)

The bioadhesive vaginal gel formulation were prepared using different polymer system to deliver the optimize drug combination effectively. During the preparation of formulation, two major issues include patients comfort ability and retention time of formulation, were given special priority. The performance and safety issues were investigated on nine different formulations on the basis of different in-vitro and in-vivo parameters study. All the prepared gel formulations were transparent, smooth, free from any grittiness and homogeneous in nature. The gel formulations were slightly yellowish in color, which may be due to the color of curcumin, and hold satisfactory yield value. Again prepared formulations reflect good spreadability, which indicate ease of application of formulations in the vaginal area. The pH of the prepared formulations were ranges within (3.8-4.4), which complies with normal pH of vagina hence, consider suitable for vaginal application & at the same might not disturb natural defense phenomenon of micro floras. Again in case of vaginal formulation viscosity is considered as an important parameter for semisolid dosage, since high viscous formulations will better adhere to the mucous wall. Hence, better will be the retention time. In this contest the viscosity of prepared formulations was found in the range of (1070-64300 Cp). The mark difference in the observed viscosity may be due to the difference in concentration and variation in grade of used polymer. Further, it has been observed that formulation prepared with single polymer shown less viscosity than the combination of polymer. Among all, F5, F8 and F9 formulation shows higher viscosity 38350cp, 53200cp & 64300cp respectively. Again the result of bioadhesion study reflects that formulations F5, F6 & F7 shows higher bioadhesion capacity as compare to others. The result of drug content study indicates that all the prepared formulations contain around 86-94% of drugs, which may be consider as a sign of good formulation. Results of the all the essential in-vitro evaluation parameters are shown in Table no-02

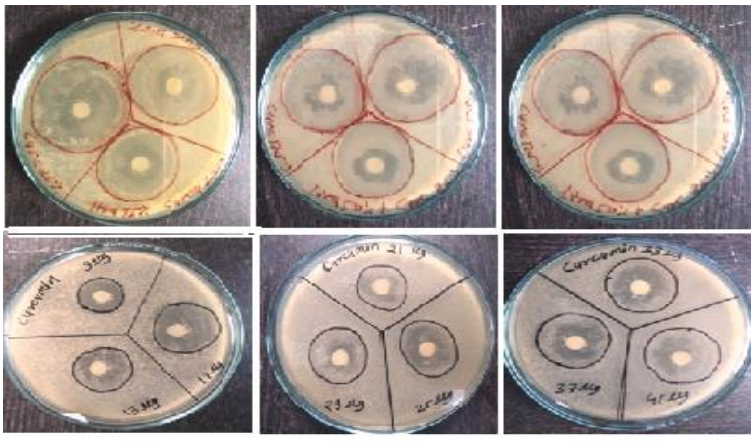
The drug release study was perform using modified Franz diffusion cell set up which reflects around 75-80% within 6-7 hrs (fig-02). RVI test were preformed to determine the safety futures of the prepared formulation. The results indicate no signs of irritation include the development of a rash, inflammation, swelling, scaling, and abnormal tissue growth was observed on applied area of rabbit, hence the formulation may consider safe.

Table 2: Result of In-Vitro evaluation parameters:

F code	Viscosity (Cp)	pH	Spreadibility (g.cm/sec)	Bioadhesion (Dyne/cm^2)	Percentage yield%	Drug content
F1	1170 ± 14.20	4.2 ± 0.03	0.533 ± 0.0252	13.275±0.651	92.59±0.888	91 ± 1.25
F2	2780 ± 09.90	4.05± 0.01	0.275 ± 0.0122	17.350±0.694	91.82±0.034	90 ± 0.80
F3	3113± 08.85	4.21± 0.02	0.141± 0.0181	23.080±0.819	93.59±0.093	86 ± 1.25
F4	29400 ± 14.50	4.2 ± 0.02	0.337±0.0230	18.025±0.397	93.25±0.051	89 ± 1.24
F5	38350 ±12.40	4.3 ± 0.05	0.433±0.0224	26.090±0.781	96.00±1.29	91 ± 1.05
F6	35800 ± 17.50	4.4 ± 0.14	0.585±0.0229	26.260±0.960	98.53±0.012	93 ± 1.28
F7	2480 ± 11.40	3.8 ± 0.12	0.522±0.0171	28.550±1.139	89.52±0.015	92 ±1.20
F8	53200 ±19.30	4.5 ± 0.35	0.639±0.0225	22.775±1.180	98.51±0.029	89 ±1.15
F9	64300 ±17.40	3.9 ± 0.03	0.742±0.129	24.62±0.029	83.15±0.031	94 ± 0.89

Table 3: Screening of antifungal activity of itraconazole and curcumin combination and estimation of FICI Value

Sl No.	Combination (ITR+CUR) µg/ml	FIC Itraconazole (ITR)	FIC Curcumin (CUR)	FICI value	Interaction
1	12 + 6.25	0.570	0.365	0.935	Antagonism
2	12 + 3.82	0.490	0.316	0.810	Antagonism
3	12 + 1.56	0.423	0.271	0.694	Antagonism
4	8 + 12.5	0.640	0.410	1.050	Antagonism
5	8+ 6.25	0.445	0.285	0.730	Antagonism
6	8 + 3.82	0.369	0.326	0.605	Antagonism
7	8 + 1.56	0.298	0.192	0.489	**Synergistic**
8	6.4 + 3.82	0.319	0.204	0.523	Antagonism
9	6.4 + 1.56	0.248	0.159	0.407	**Synergistic**
10	4 + 19	0.718	0.460	1.17	Antagonism
11	4 + 12.5	0.515	0.330	0.840	Antagonism
12	4 + 6.25	0.320	0.205	0.525	Antagonism



Initial screening of zone of inhibition of curcumin and fluconazole by disk diffusion method

Fig 1: Initial screening of antifungal activity

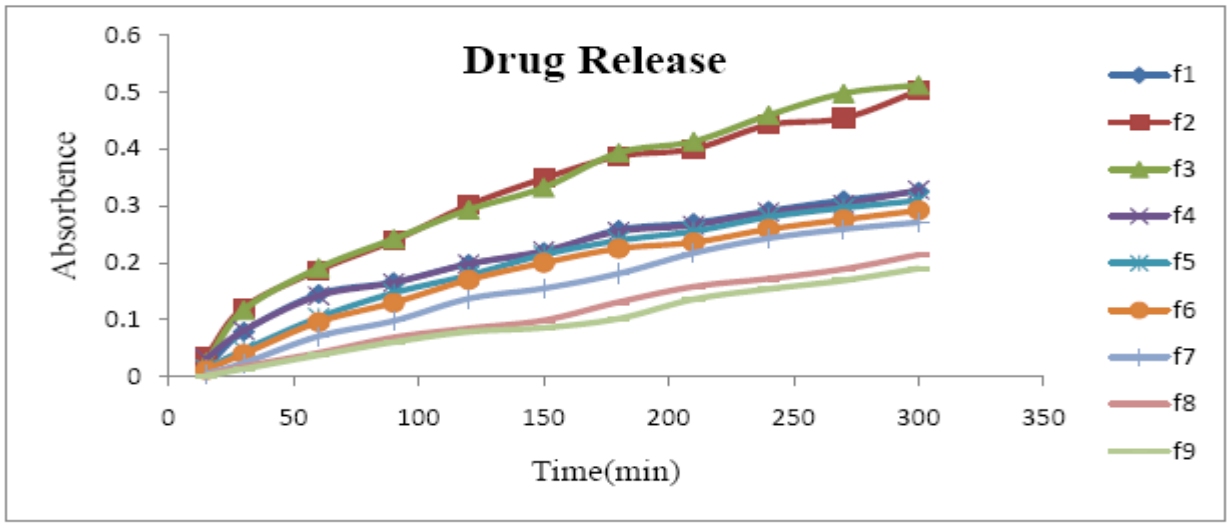


Fig 2: Graphical representation of drug release study of the prepared formulations

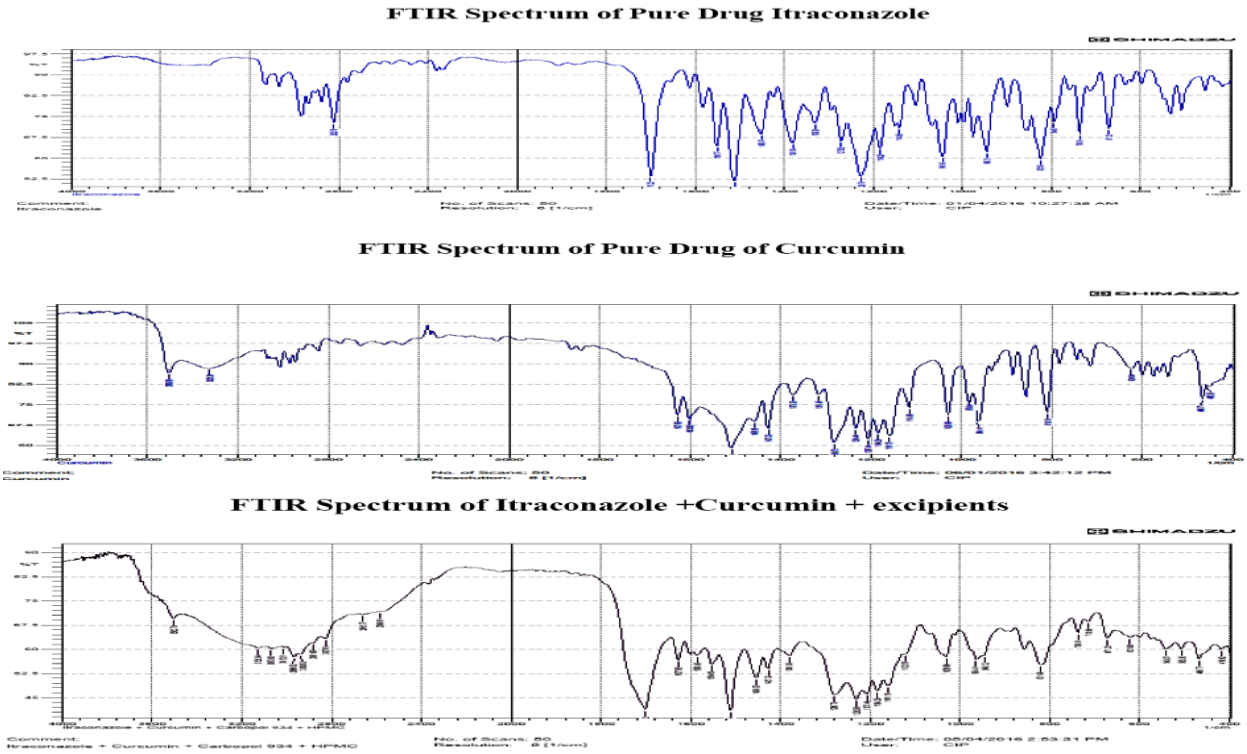


Fig 3: Graphical representation of FTIR study of pure drugs (curcumin & Itraconazole) and combination of drug and polymer.

CONCLUSION:

The presence investigation reveals that, bioactive phytochemical curcumin leads to increases the antifungal effect of itraconazole, which may be due to the mechanism synergism. Based on the results of in-vitro and In-Vivo evaluation it can be conclude that all the prepared formulations fulfill the required criteria to be a suitable vaginal formulation, however, formulation F5 (carbopol p934 & HPMC in a ratio 1:1) shown better performance in respect of their bioadhesion capacity, property of spreadibility and drug release study, that may facilitate the vaginal

application and demand to increase poor patient compliance. The in vivo animal studies indicate no sign of irritation. Further study may be carried out the establish the formulation for commercial application.

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