In vitro Evaluation of Miconazole Nitrate Loaded Mucoadheshive Patches for Effective Management of Oral Candidiasis

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Abstract

Miconazole nitrate bearing patches were prepared to deliver drug in buccal cavity for local action for effective management oral candidiasis. It were prepared by solvent cast method employing the varieties of polymers ratios and then studied for their % drug entrapment efficiency, patch thickness, folding endurance, tensile strength and moisture content and uptake. Furthermore, the in vitro antifungal activity of the formulated mucoadhesive Patches was significantly ($p<0.05$) higher to the reference marketed formulation.

Keywords: Drug delivery, patches; candidiasis, mucoadhesive, antifungal activity.

1.0 INTRODUCTION

Mucoadhesive drug delivery systems play a very important role in site specific delivery of drug for local and systemic effect. Some polymers either natural or synthetic have properties to adhere on biological mucus membrane of the GIT system (Khurana et al., 2000). The bio-adhesion properties of polymers can utilize to formulate the drug delivery system that are capable to adhere on the mucus membrane and deliver drug for longer period of time in sustained manner (Nafee et al., 2003). Bioadhesive drug delivery systems are adhering on the mucus membrane by the interfacial force. These drug delivery system can be utilize to deliver drug site specifically by buccal, oral, veginal, rectal, ocular, and nasal route. Bioadhesive drug delivery system are beneficial for drugs which absorb from specific site, it can avoid first pass effect and minimize the drug loss by degradation. Candidiasis is caused by a fungus it is a type of yeast called candida. Normally it is present in oral cavity without any harmful effect but when it invade the mucosa and grow under its lining then it cause the infection. It can infect the mouth and throat. The infection caused by candida in the mouth and throat called oral thrush or orophryangeal candidiasis. Candidiasis is characterized with white patches on tongue, mouth, and throat and in the inner lining of cheeks, which result in loss of taste, pain and difficulty in eating and swallowing (Akimoto et al., 2003; Baert et al., 2012). Miconazole nitrate is a imidazole derivates has potent antifungal activity that is used in the treatment of ring worm infection, pityriasis, vesicolor and infection caused by yeast. Miconazole nitrate is a choice of drug that can use to treatment of candidiasis in the form of oral gel or given by oral and IV route.

In the treatment of oral candidiasis, the oral gel, tablet and IV dose of antifungal drug is administered which distribute systemically in whole body and very low concentration of administered dose is reached in oral cavity that may very low for therapeutic effect. Unwanted distribution may precipitate side effect and also increase the cost and duration of treatment. In this present work, the main objective was to retain the effective drug concentration in the oral cavity to treat the infection caused by candid yeast. In it was proposed to prepare the mucoadhesive patches which that adhere to mucosa of oral cavity and release the antifungal drug in sustained manner that maintain the high concentration of drug in oral cavity for longer time.

2.0 MATERIAL AND METHODS

2.1 Material

Miconazole nitrate was purchased from Cipla Ltd. (Mumbai, India). Hydroxy propyl methyl cellulose, HPMCK15M, Poly vinyl pyrolidone (PVPK30) and Sodium carboxy methyl cellulose (NaCMC), PEG 400, Agar were purchased from Himedia pvt Ltd, Mumbai, India. Miconazole nitrate was obtained as gift sample. All other reagents were of analytical grade were purchased from Himedia Pvt Ltd and Central Drug House (CDH), Mumbai India.

2.2 Method

The solvent casting method was used to formulate the miconazole containg polymeric patches. The polymers HPMC K4M, HPMC K15M and sodium carboxy methyl cellulose (NaCMC), was dissolved in double distilled water. Drug (10mg) was added in the above polymeric solution. Drug and polymeric solution was stirred 1hr to form homogeneous solution at 60°C. PEG 400 as a plasticizer was added after cooling of the polymeric solution. A fixed volume of solution was poured in the petri dishes (9.0 cm of radius) that were previously wetted with glycerin. All the petri dishes were kept under hot air oven at 30°C for the purpose of drying. After complete drying the patches from the petri dishes were removed and cut in the pieces of 2 cm^2 and stored in air tight container .

Table 1: Formulation of patches

Formulation Code	Drug Concentration	HPMC-K15M	Na-CMC	PVA	PEG 400
BPT-1	50	600	300	200	0.15
BPT-2	**50**	**800**	**300**	**200**	**0.15**
BPT-3	50	1000	300	200	0.15
BPT-4	**50**	**800**	**400**	**200**	**0.15**
BPT-5	50	800	500	200	0.15
BPT-6	50	800	400	300	0.15

BPT-7	**50**	**800**	**400**	**400**	**0.15**
BPT-8	50	800	400	500	0.15
BPT-9	**50**	**800**	**400**	**400**	**0.20**
BPT-10	50	800	400	400	0.25

2.3 In vivo study for Oral Candidiasis

In vivo performance of drug bearing formulations was performed for their antifungal effect. The *in vivo* study protocol involves the safety and efficacy evaluation of prepared formulations and its ultimate fate inside animal body. In this context, the present topic deals with the biodistribution and pictorial evaluation of drug loaded patches formulation in oral cadidiasis bearing rabbit. Further, biodistribution study was also performed at a predetermined dosage regimen.

The studies were carried out as per the guidelines of CPCSEA, Ministry of Social Justice and Empowerment, Govt. of India, following the protocol approved by the Institutional Animals Ethics Committee of SRK University, Bhopal, MP. Animals were housed in groups of six with free access to food and water. Proper care of animals was taken during the study period.

Induction of candidiasis

An ideal animal model making for oral candidiasis is required to get a standard means that can generate, and controlled the data on the identification and treatment of the disease. The available animal models are revealing the pathogenesis for oral candidiasis from the host and the yeast points of view. Optimized formulations were tested for their antifungal activity on animal model using male rabbit. Firstly the candidiasis infection was induced by giving few drops of culture of Candida albicans orally. Animals are observed for infection in their oral cavity. Culture of Candida albicans was given orally for three days. Infected rats were housed individually in wire bottom cages and were provided with food and water. The candidiasis infection was observed on the rats after 3 days. To confirm the candidiasis infection, the oral mucosa of rat was scraped on the 4^{th} day. The scrapped mucosa was inoculated on dextrose agar media plates. The inoculated plates were incubated at 27 ^{o}C for 48 hr. The colonies were measured after incubation.

Anti microbial Zone of Inhibition study

The zone of inhibition studies was performed to check the antifungal effect of the selected formulations i.e. BPTH-1, BPTH-2, BPTH-3 and BPTH-4 and compare with standard plain drug solution against Candida albicans. Nutrient agar solution was prepared and sterilized by autoclave to remove any microorganism contaminations. The prepared agar media (15.0 mL) was mixed with Candida albicans suspension (0.5 mL) to seed it with media. The prepared mixture was poured in previously sterilized Petri discs (9.0 cm) under aseptic condition and allowed to solidify after covering the Petri discs with its cover. The 1.0 cm^2 of the selected formulation i.e. BPTH-1, BPTH-2, BPTH-3, BPTH-4 and standard drug solution soaked Wattman filter paper were putted in the petri discs by means of previously sterile forceps. The plates were incubated at 27ºC for next 48 hr by using BOD incubator (Labhosp, India). After 48 hr plates were removed from incubator and measure the diameters of the zones of inhibition.

In-vivo antimicrobial study on animals

The experiments were performed using films with the optimum formulation. Forty-five male rabbits weighing 2.5–3.0 kg were fed under the same laboratory conditions. The protocol was approved by the Ethical Committee of the institute, SRK University. Rats were randomly divided into five groups (n ¼ 9). To establish the oral candidiasis model, all the rabbits were given drops of culture of Candida albicans on the oral cavity. Treatment was initiated after infection initiation. The model group was not treated, while the other four groups were given Plain Drug, BPTH-2 respectively films were treated for 30 min once a day. All the films (1 cm^2) were attached on the oral mucosa. On the 4^{th} day, the oral mucosa of rat was scraped and was cultivated in sabouraud dextrose agar media plates. The inoculated plates were incubated at 27 ^oC for 48 hrs. The colonies were measured after incubation. On the 4th day, treatment was initiated by topical application to the infected sites with prepared formulation of patches for another 4 days. On the 8 day the oral mucosa was again scraped and cultured on sabouraud dextrose agar plate respectively and further treatment was done. The inoculated plates were incubated at 27ºC for 48 hrs and examined for growth of colonies. The number of colonies were counted using Cintex colony counter

Table 2: Antimicrobial activity of different formulation comparing with plain drug

Formulation Code	Organism used	Zone of Inhibition
Plain drug	Candida albicans	17.8±1.3 mm
BPTH-1	Candida albicans	18.3±0.8 mm
BPTH-2	Candida albicans	18.5±0.9 mm
BPTH-3	Candida albicans	19.5±1.5 mm
BPTH-4	Candida albicans	21.2±1.2 mm

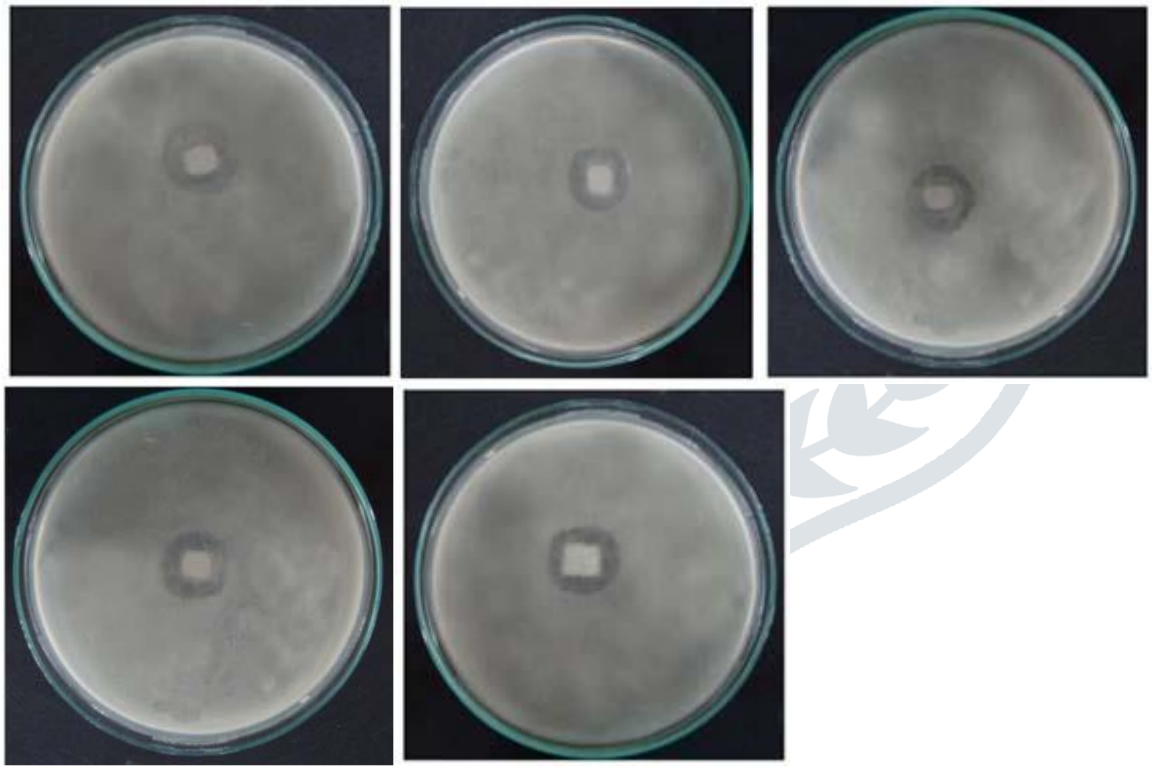


Figure 1: Agar plate showing zone of inhibition for control and optimized formulation

Table 3: Antifungal activity on rat oral mucosa

Formulation code	Colony count after Fungal infection	Colony count on 8th day after treatment	Colony count on11th day after treatment
Plain drug	>125	105	95
BPTH-1	>125	78	35
BPTH-1	>125	85	32
BPTH-3	>125	73	27
BPTH-2	>125	62	19

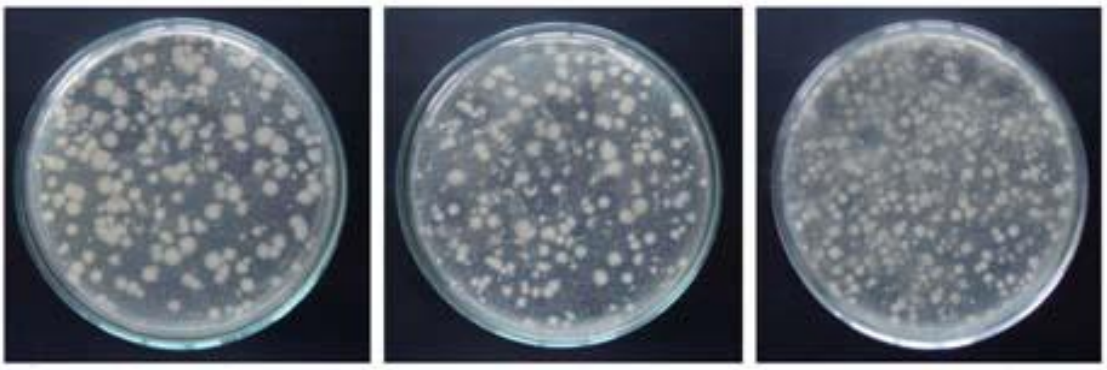


Fig. 2: Agar plate showing inhibition of colony count with formulation BPTH-1 at day 4, 8 and 11

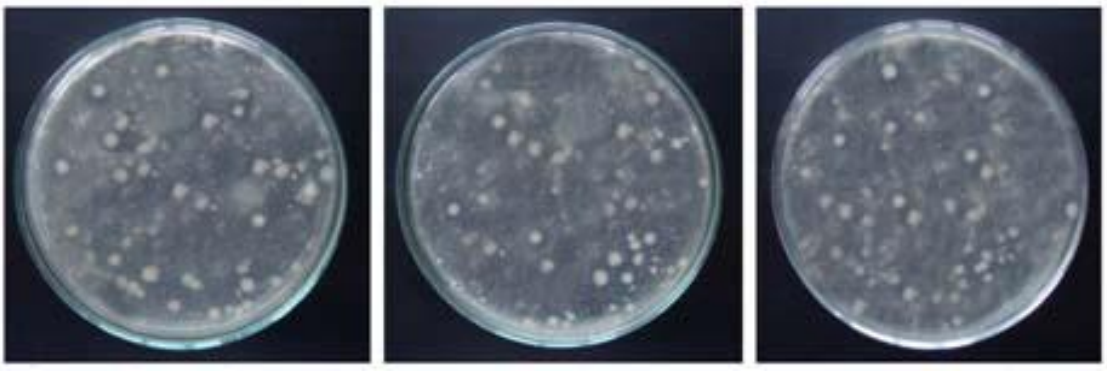


Fig. 3: Agar plate showing inhibition of colony count with formulation BPTH-1 at day 4, 8 and 11

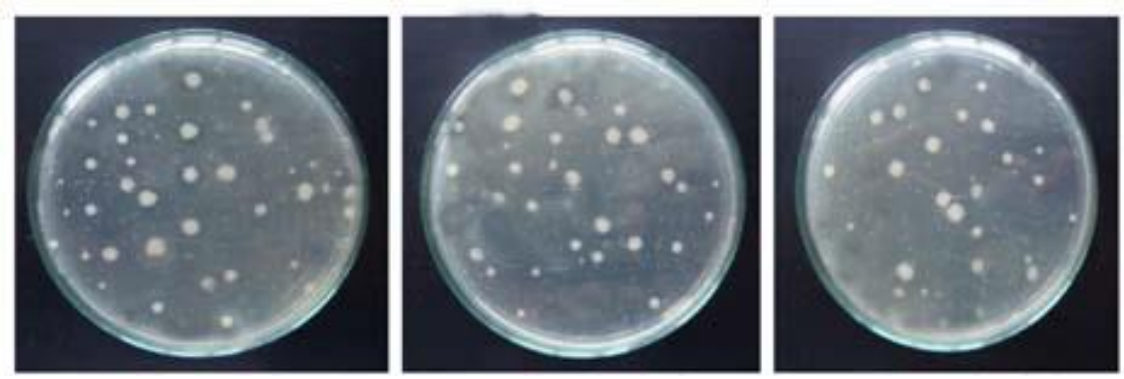


Fig. 4: Agar plate showing inhibition of colony count with formulation BPTH-3at day 4, 8 and 11

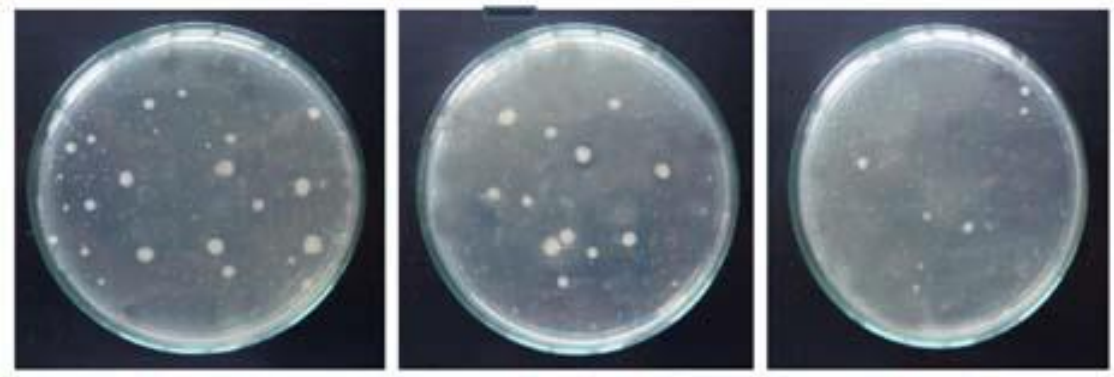


Fig. 5: Agar plate showing inhibition of colony count with formulation BPTH-2 at day 4, 8 and 11

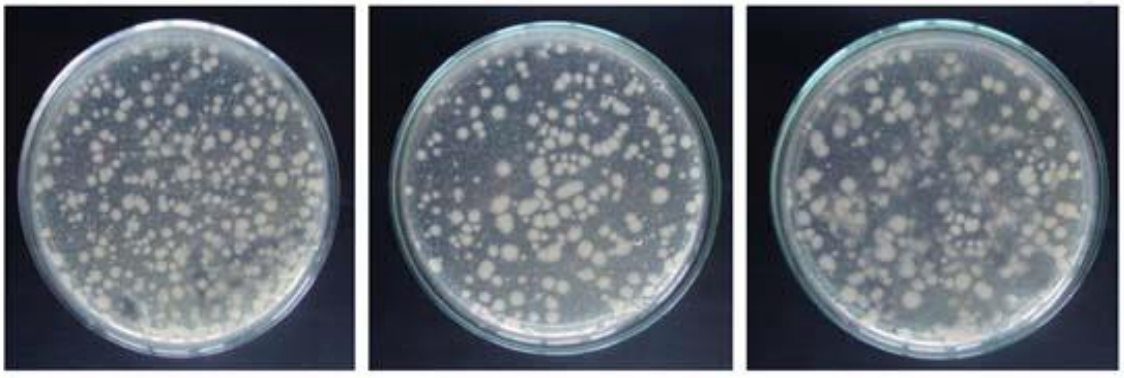


Fig. 6: Agar plate showing growth of colony with plain drug.

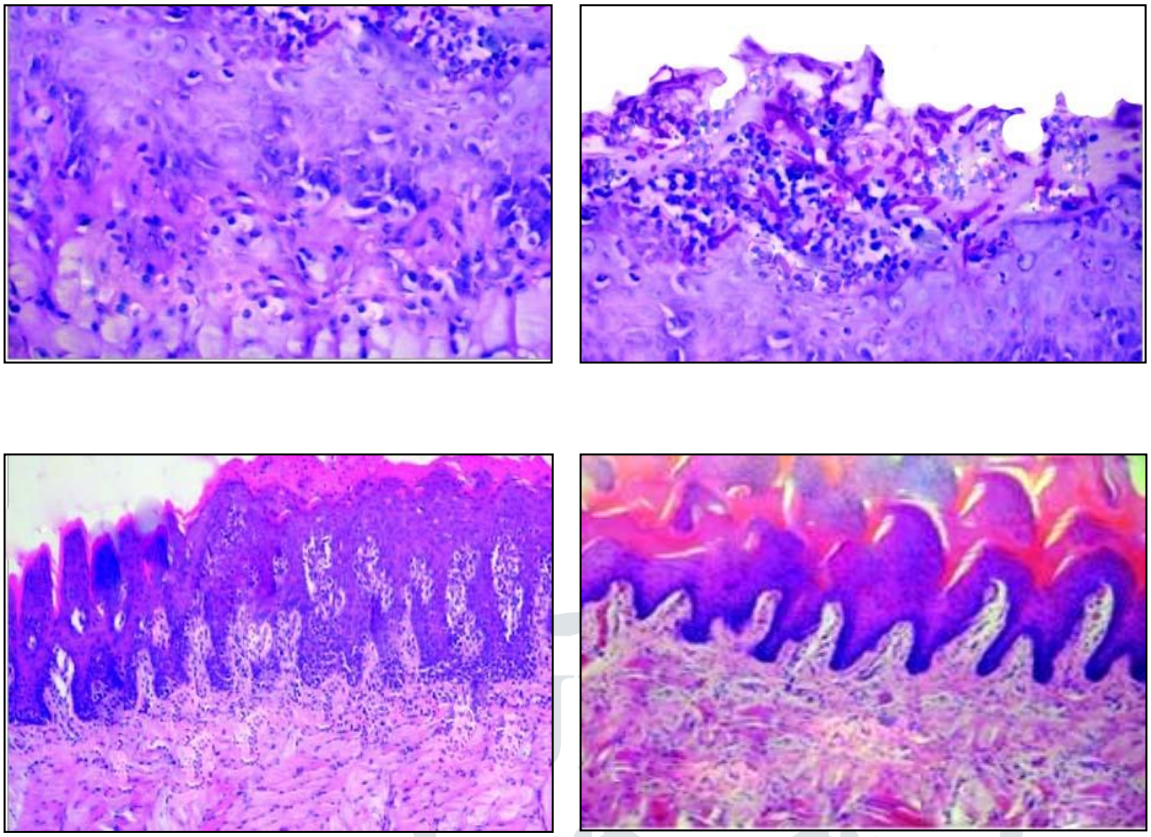


Fig. 7 Histopathology image of oral cavity (tongue) showing level of candidiasis infection after administration of BPTH-1, BPTH-1, BPTH-3 and BPTH-2

Drug Distribution Study

The tissue distribution of plain drug, and prepared drug loaded patches formulations i.e. plain drug and BPTH-1, BPTH-2, BPTH-3 and BPTH-4 was studied following their topical oral administration. The candidiasis induced rabbits were divided in five groups each consisting of 3 animals. Plain drug, and prepared drug loaded formulation i.e BPTH-1, BPTH-2, BPTH-3 and BPTH-4 were applied to animals of first, second, third, fourth group, and fifth group respectively at random in the buccal mucosa. After 1, 4, 8 and 24h of administration, the rabbit were sacrificed by cervical dislocation, and their tissues such as stomach, intestine, liver, were excised, washed quickly with cold water to remove surface blood and isolated organs were again washed with the ringer solution and then one gram of each organ was homogenated using tissue homogenizer with 2 mL PBS (pH 7.4). In the case of organs weighing less than one gram, the whole organ was used, and 150µL of tissue homogenate was taken, and equal volume of acetonitrile was added

(kept for 30 min). The mixture was centrifuged at 5000 rpm for 10 minutes and supernatant was filtered through 0.22 µm membrane filter. The samples were analyzed by HPLC for drug concentration.

Saliva was collected to estimate the drug concentration in the oral cacity. The sample of saliva was collected just before before sacrificed the animals. Blood samples (1 mL) were also obtained in duplicate by orbital plexus from rabbits in pre-weighed heparinized tubes and same procedure was applied as discussed above for estimation of DOX. The biodistribution of plain drug, drug loaded formulation in each organ was calculated as a percentage of the injected dose per gram of the tissue (%ID/g).

The following formula was used to calculate percentage of the administered dose per gram of the tissue (% ID/g).

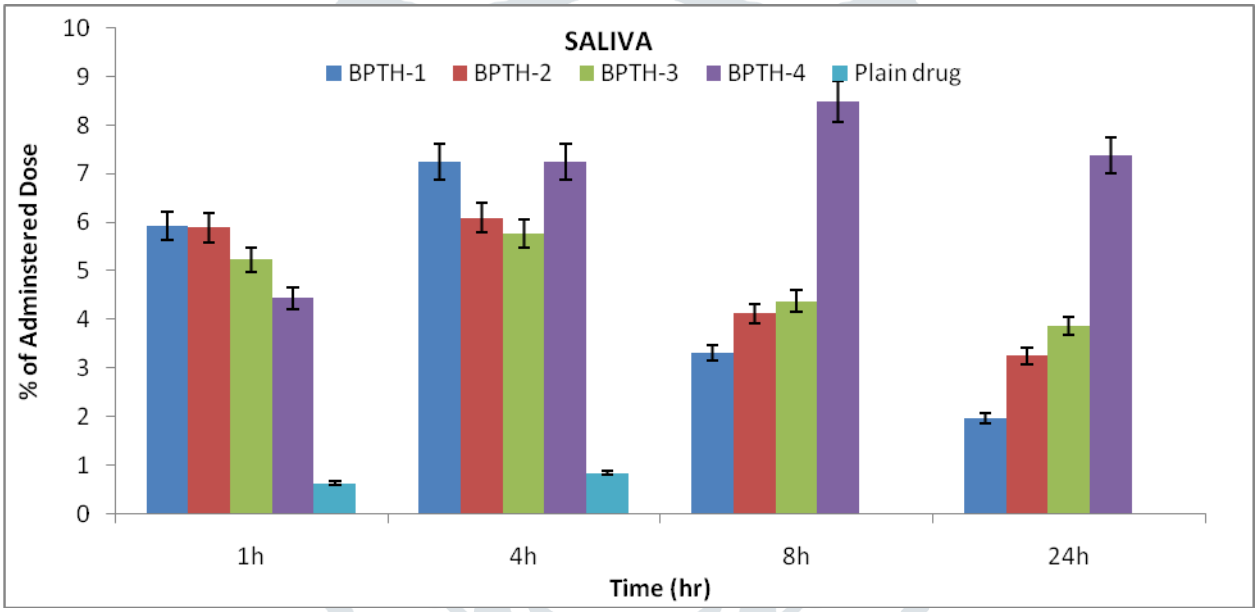
$$\%\,\text{ID per gram of tissue/organ} = \frac{\text{Counts (in Kcpm)} X 100}{\text{Weight of sample organ (g)} X \text{ total counts injected}}$$

Table 4 : Bio-distribution of drug from administered plain drug and drug loaded patches formulations into rats at three different time points

Organ/tissue	Formulation/Drug	Percent Administered Dose/g of organ			
		1h	4h	8h	24h
Saliva	Plain drug	0.64±0.92	0.85±0.41	ND	ND
	BPTH-1	5.93±0.39	7.24±0.67	3.32±0.43	1.98±0.29
	BPTH-1	5.89±0.32	6.09±0.24	4.12±0.28	3.25±0.41
	BPTH-3	5.23±0.16	5.76±0.15	4.38±0.42	3.87±0.22
	BPTH-2	4.45±0.07	7.24±0.15	8.48±0.37	7.37±0.52

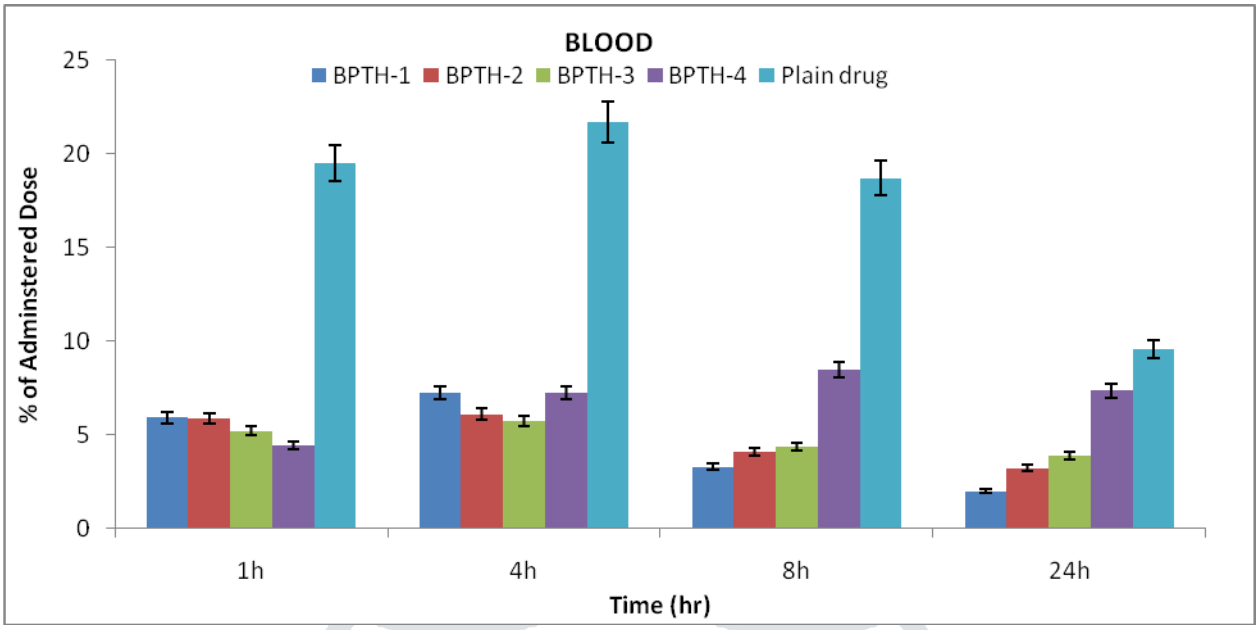
Organ/tissue	Formulation/Drug	Percent Administered Dose/g of organ			
		1h	4h	8h	24h
Blood	Plain drug	19.51±2.03	21.72±1.34	18.71±2.31	09.57±0.07
	BPTH-1	2.25 ± 0.21	2.68 ± 0.34	3.95 ± 0.24	1.58 ± 0.09
	BPTH-1	2.16 ± 0.13	2.39 ± 0.14	3.86 ± 0.18	1.39 ± 0.12
	BPTH-3	2.19 ± 0.32	2.16 ± 0.28	3.62 ± 0.11	1.15 ± 0.09
	BPTH-2	1.79 ± 0.33	1.96 ± 0.18	2.02 ± 0.18	2.12 ± 0.35
Stomach	Plain drug	42.12±0.75	0.17±0.35	0.48±0.17	ND
	BPTH-1	5.93±0.39	7.24±0.67	3.32±0.43	1.98±0.29
	BPTH-1	5.89±0.32	6.09±0.24	4.12±0.28	3.25±0.41
	BPTH-3	5.23±0.16	5.76±0.15	4.38±0.42	3.87±0.22
	BPTH-2	4.45±0.07	7.24±0.15	8.48±0.37	7.37±0.52
Small Intestine	Plain drug	27.93±2.13	2.24±3.13	0.53±0.66	ND
	BPTH-1	1.81±0.32	2.26±0.18	2.59±0.22	2.98±0.12
	BPTH-1	1.79±0.41	2.23±0.15	2.53±0.32	2.93±0.15
	BPTH-3	1.58±0.23	1.96±0.15	2.28±0.22	2.86±0.09
	BPTH-2	1.13±0.16	1.56±0.35	2.18±0.41	2.67±0.07
Liver	Plain drug	8.27±0.76	5.33±0.63	5.16±0.84	4.5±0.52
	BPTH-1	0.53±0.06	2.16±0.14	2.35±0.53	3.1±0.22

Organ/tissue	Formulation/Drug	Percent Administered Dose/g of organ			
		1h	4h	8h	24h
	BPTH-1	1.23±0.08	1.72±0.15	3.89±0.15	3.7±0.31
	BPTH-3	2.28±0.03	2.47±0.04	4.33±0.13	3.2±0.13
	BPTH-2	2.28±0.13	3.47±0.16	3.78±0.15	4.2±0.21



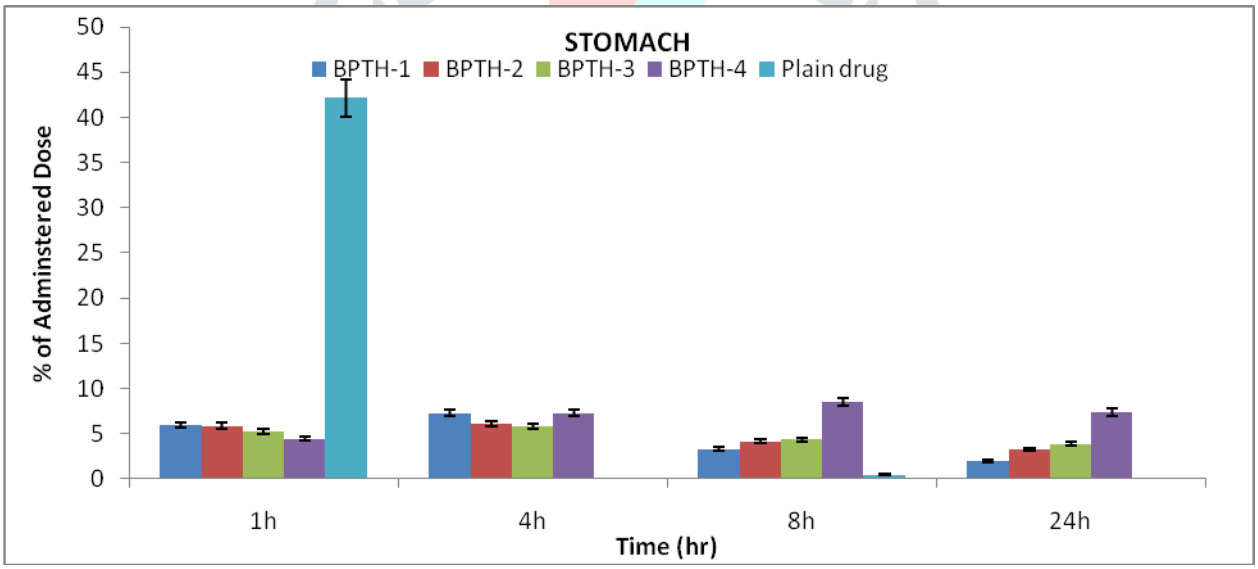
Values represents mean±SD (n=6);

Fig. 8: Drug distribution in saliva from administered formulations miconazole nitrate, miconazole nitrate loaded buccal patches, BPTH-1, BPTH-1, BPTH-3 and BPTH-2.



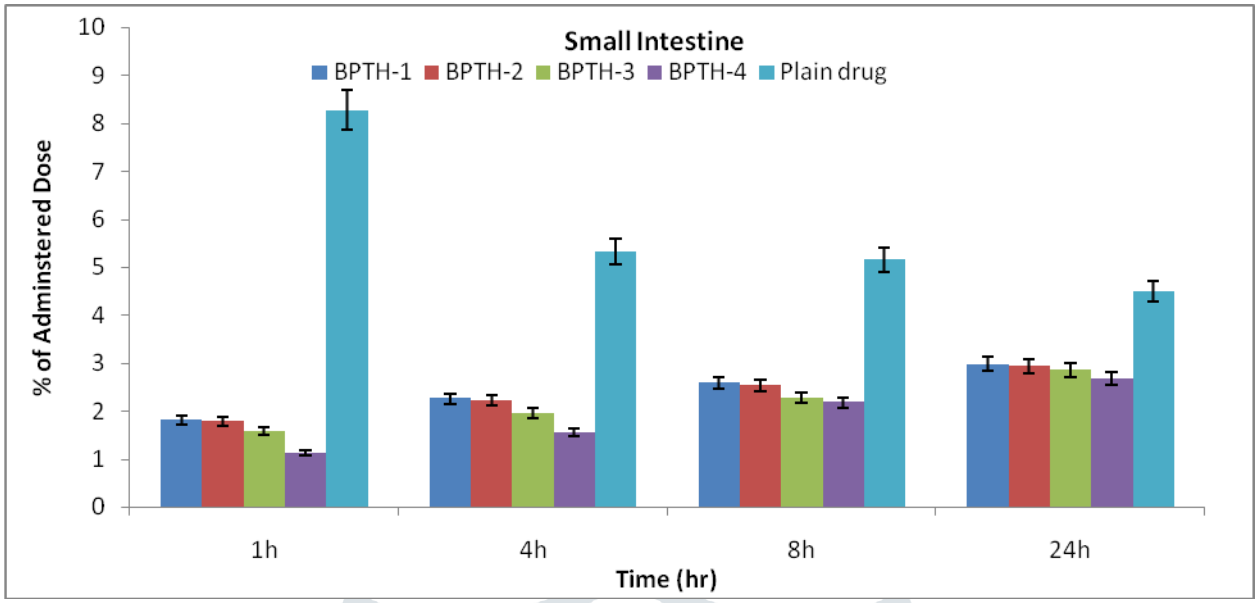
Values represents mean±SD (n=6);

Fig. 9: Drug distribution in blood from administered formulations miconazole nitrate, miconazole nitrate loaded buccal patches, BPTH-1, BPTH-1, BPTH-3 and BPTH-2.



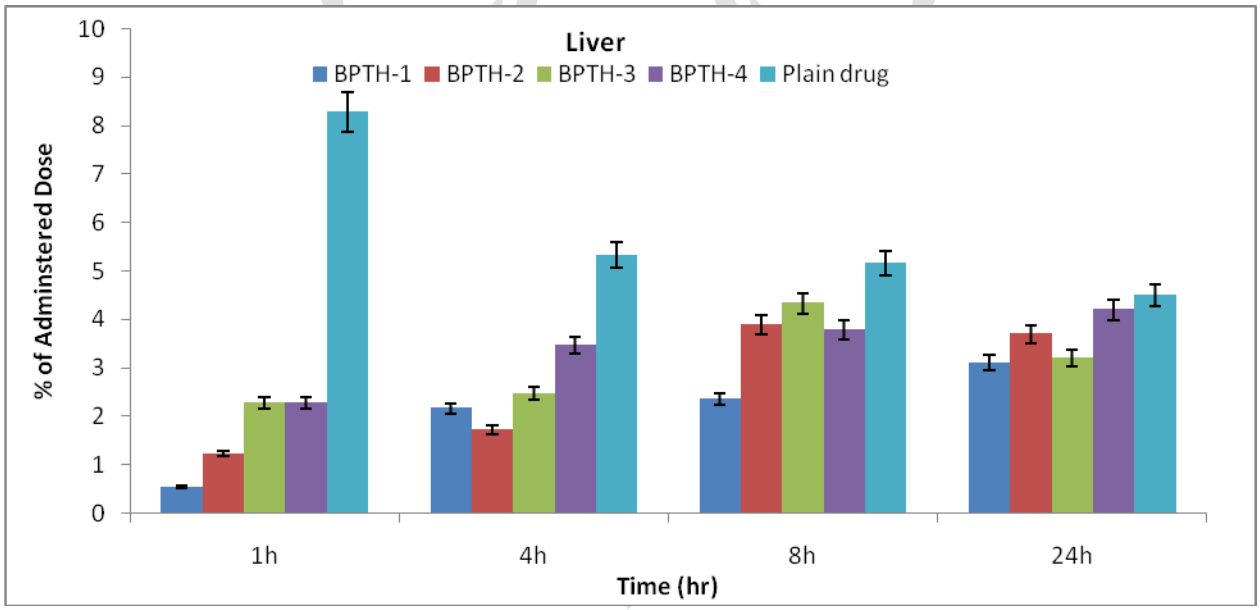
Values represents mean±SD (n=6);

Fig. 10: Drug distribution in stomach from administered formulations miconazole nitrate, miconazole nitrate loaded buccal patches, BPTH-1, BPTH-1, BPTH-3 and BPTH-2.



Values represents mean±SD (n=6);

Fig. 11: Drug distribution in small intestine from administered formulations miconazole nitrate, miconazole nitrate loaded buccal patches, BPTH-1, BPTH-1, BPTH-3 and BPTH-2.



Values represents mean±SD (n=6);

Fig. 12: Drug distribution in liver from administered formulations miconazole nitrate, miconazole nitrate loaded buccal patches, BPTH-1, BPTH-1, BPTH-3 and BPTH-2.

Histopathology study:

The selected animals were anesthetized and sacrificed after antimicrobial study on day 12 for histopathological examination (Karavana et al. 2011). This study was performed to examine the depth of the infection and also

assess the effect of the drug and drug loaded patches to treat the infection. On the basis of previous study data i.e. antimicrobial studies the formulation BPTH-4 was selected for the histopathalogical study and their data were compare with the plain drug solution and control group. The tissues from oral cavity were cut and removed that were washed with Ringer's solution. After washing they were cut into small pieces and dried on tissue paper. The tissues were fixed in 4% v/v neutral aqueous paraformaldehyde and further 4 mm-thick tissue piece were entrenched in paraffin blocks. The sections (5-µm of thickness) were cut from each of the blocks and then sections were stained with periodic acid-Schiff (PAS), by Grocott's silver stain method, which reveal the confirmation of the degree of fungal infection and the depth of penetration in the tissue. The obtained piece was fixed on the glass slides using solution of the egg albumin. Prepared slides were examined under a light microscope with 100X magnification and taken images of the tissue which are fixed on the glass slide. The therapeutic effect was evaluated by the density of the neutrophils in the oral sub-epithelial tissue.

3. Result and Discussion

The yeast strain Candida albicans ATCC 90028 cultures was used to check the antimicrobial potential of prepared patch. Agar diffusion method was used to determine the antimicrobial sufficiency of the prepared patches. The strain was subcultured on dextrose Agar media. The optimized formulation PBTH-1, BPTH-2, BPTH-3 and BPTH-4 and a standard drug solution containing miconazol nitrate were placed on the dextrose agar plate. Agar plates were cultured with the candida albicans ATCC 90028 before testing. The patches of $1cm^2$ containing miconazole nitrate were placed on agar plate and the marketed formulation was also placed on agar plate that covers $1cm^2$ area. After covering of plates, the plates were incubated in the incubator at 37±0.5°C for 24 h. The growth zone inhibition was measured as mean SD±, n=6. The Statistical analysis was performed to compare the prepared formulation from marketed patches. The selected formulation such as BPTH-1, BPTH-2, BPTH-3 and BPTH-4 where revealed 18.3±0.8 mm, 18.5±0.9 mm, 19.5±1.5 mm, 21.2±1.2 mm and 17.8±1.3 mm respectively. The formulation BPTH-4 was given very significant result with zone of inhibition of 21.2±1.2mm when compare with other selected patches formulation (Table 1).

The data obtained from antifungal activity on oral cavity of animal rat model given in Table 2. The candidiasis infection was developed successfully on the oral cavity of the rat after 3 days by giving fungal suspension.

After induction of oral candidiasis which was confirm by incubating the scrapped mucosa sample and count the colony developed on agar plates. More than 150 similar colony confirm the infection. The formulations were administered after 5th day of incubation and the mucosa sample was scrapped after predefined time interval. It was observed that PBT-2, PBT-4, BPTH-3 and BPTH-4 formulation was showed reduction in colony count in comparison to plain drug sample. And within the PBT-2, PBT-4, BPTH-3 and BPTH-4 the BPTH-4 formulation give better result in comparison to PBT-2, PBT-4, BPTH-3. The treatment was carried out by repeating sample administered for next 4 days. The colony count for PBT-2, PBT-4, BPTH-3 and BPTH-4 was78, 85, 73 and 62 respectively

After 9th day of treatment the samples were again scrapped and checked for the candidiasis colony growth. On 9th day of treatment the colony count for PBT-2, PBT-4, BPTH-3 and BPTH-4 was 35, 32, 27, 19 respectively (fig 2 to 6).

The antifungal and in-vivo study results revealed that prepared formulation PBT-2, PBT-4, BPTH-3 and BPTH-4 revealed effective antifungalactivity. However, within all the patches PBT-9 formulation was given significant antimicrobial activity against candidiasis infection.

Bio-distribution study of miconazole loaded formulations after 1, 4 ,8 and 24h of buccal administeration in different organs of candidiasis induced rabbit was performed to assess the what amount or concentration of miconazole nitrate was supplied or maintained from the selected polymeric patches formulations to the infected oral cavity and other organ in the i*n vivo.* The purpose of the formulating the buccal polymeric patches was to maintain sufficient concentration of antifungal drug i.e. miconazole nitrate to the infected oral cavity for a longer period of time. The prepared buccal patches have sufficient mucoadhesive properties which help to adhere with buccal mucosa. Drug concentration observed was reported as percent injected dose per gram of organ/tissue. The histopathologically study was also performed to further confirm the data obtained by other study. As expected the selected formulations BPTH-1, BPTH-2, BPTH-3 and BPTH-4 formulation were maintain the drug concentration in the oral cavity when compare with plain drug solution. This was due to the mucoadhesive and sustained release properties of prepared buccal patches. In comparison with other selected formulation BPTH-4 was given significant result and maintains the sufficient concentration in the oral cavity

for longer period of time (Figure 2 and figure 6). histopathological examination was performed to examine the depth of the infection and also assess the effect of the drug and drug loaded patches to treat the infection. The tissues from oral cavity were cut and removed that were washed with Ringer's solution. After washing they were cut into small pieces and dried on tissue paper. The tissues were fixed in 4% v/v neutral aqueous paraformaldehyde and further 4 mm-thick tissue piece were entrenched in paraffin blocks. The sections (5-µm of thickness) were cut from each of the blocks and then sections were stained with periodic acid-Schiff (PAS), by Grocott's silver stain method, which reveal the confirmation of the degree of fungal infection and the depth of penetration in the tissue. The obtained piece was fixed on the glass slides using solution of the egg albumin. Prepared slides were examined under a light microscope with 100X magnification and taken images of the tissue which are fixed on the glass slide. On the basis of previous study data i.e. antimicrobial studies the formulation BPTH-4 was selected for the histopathalogical study and their data were compare with the plain drug solution and control group. It was revealed that the fungal infection clearly disappears in case of BPTH-4 formulation when it was compared with plain drug solution and control group (fig. 7).

The drug was estimated in the oral cavity by taking the sample of saliva with suitable time interval. In case of saliva, drug concentration in 1, 4, 8 and 24 hr was revealed 4.45±0.07, 7.24±0.15, 8.48±0.37 and 7.37±0.52% with BPTH-4 as optimized formulation. The formulation BPTH-4 was maintained the concentration constantly for 24 hr. In case of plain drug, the drug was disappear before 1 hr and was found in very minute concentration of 0.64±0.92% and it was become not detectable after 4 hr. The drug concentration was maintained by the other formulation like BPTH-1, BPTH-2, and BPTH-3 in first 1hr to 8hr but the concentration was decline after 8hr (table 4 and fig 3).

The drug concentration in blood with BPTH-4 was found 1.79 ± 0.33, 1.96 ± 0.18, 2.02 ± 0.18 and 2.12 ± 0.35 % in 1, 4, 8 and 24 hr respectively. And in case of plain drug solution it was found that drug concentration in blood was reached with 19.51±2.03, 21.72±1.34, 18.71±2.31 and 09.57±0.07 respectively in 1, 4, 8 and 24 hr (fig 9 and table 4)

In case of drug concentration in stomach, when plain drug solution was given orally to the animal drug concentration was found maximum of 42.12±0.75% of administered dose in first 1 hr. Drug was transit

immediately from oral cavity to stomach in case of oral drug solution administration. In case of optimized formulation i.e. BPTH-4, drug concentration was found 4.45±0.07, 7.24±0.15, 8.48±0.37 and 7.37±0.52 respectively in stomach in 1, 4, 8 and 24 hr. The drug concentration in first 1 hr after administration of BPTH-1, BPTH-2, BPTH-3 was found 5.93±0.39, 5.89±0.32 and 4.45±0.07 respectively which was found decline in 24hr (table 4 and figure 11). In case of small intestine, drug concentration was not significantly found after administration o BPTH-1, BPTH-2, BPTH-3 and BPTH-4 formulation except plain drug solution. It was found 27.93±2.13% of drug was reached in small intestine with plain drug solution administration in first hours and was found not significantly in 4, 8 and 24 hr (fig 11 and table 4)

In liver, drug concentration 2.28±0.13, 3.47±0.16, 3.78±0.15 and 4.2±0.21% was found in case of BPTH-4 in 1,4,8 and 24 hr of drug administration. And in case of plain drug administration, it was found 8.27±0.76, 5.33±0.63, 5.16±0.84 and 4.5±0.52% of drug in liver in 1, 4, 8 and 2 hr (Table 4 and figure 12).

4. References

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