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Formulation and *In-vitro, In-vivo* evaluation of Periodontal Films containing Metronidazole and Minocycline HCl

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Abstract

Local delivery devices are designed to deliver the drug locally into periodontal pocket. Metronidazole is a nitroimidazole used to treat protozoal infections. Solvent casting technique was used for the preparation of periodontal films using chloroform and dichloromethane in (1:1) mixture using hydroxypropyl cellulose (HPC), hydroxypropylmethyl cellulose K4M (HPMC K4M), polymethyl methacrylate (PMMA1, 20,000) in chloroform and dichloromethane (1:1) mixture, dibutylphthalate (10% v/w of that of polymer), glycerol (20%v/v) as plasticizers and Eudragit L-100 as polymer. FTIR and UV spectroscopic methods revealed no interaction between Metronidazole and polymers. These films are evaluated for thickness, Folding endurance, texture analysis, tensile strength, drug content, mucoadeshion stability studies and biodegradable ability. Data of *In vitro* release of films reveals that F1 formulation has maximum drug release. *In-vitro* anti bacterial activity and *in vivo* studies also performed for all the formulations. From the study we can conclude that formulation F1 has good stability and best formulation for further study.

Keywords: Metronidazole, periodontal films, local delivery, *in-vitro* release, *in- vitro* anti bacterial activity, *in-vivo* evaluation.

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1. Introduction

The word periodontal literally means "around the tooth". Periodontitis is a chronic bacterial infection that affects the gums and bones supporting teeth. Periodontal disease can affect one tooth or many teeth. Periodontitis is a disease associated with periodontium in which irreversible step of loss of attachment of teeth occurs [1]. The effective use of antimicrobial agent for the treatment of periodontal disease requires an adequate drug concentration at the site of action and a means to maintain that level for a sufficient duration to allow the agent to act. Topical applications like

mouthwashes, dentifrices and gels have been successfully tried in controlling the microbial plaque. Topical agents follows an exponential concentration profile while blood and crevicular fluid levels remains at zero, initial salivary concentration reach levels 20 to 50 times bactericidal levels, following expectoration, salivary drug level rapidly fall to approximately one tenth of their initial concentration. Topical agent fail to penetrate deep into periodontal pockets, hence their effectiveness is limited to supragingival areas. So to overcome all these limitations various controlled drug delivery systems, administrating therapeutic levels of antibacterial agents directly into periodontal pocket have been tested as a way to minimize total body dosage and resulting side effects and to maintain therapeutic drug levels in the gingival crevicular fluid [2,3].

The microbiological treatment of periodontitis is through either the use of systemic antibiotics or localizesed [4]. In the systemic use, large doses must be taken in order to achieve sufficient concentrations in the gingival crevicular fluid of the periodontal pocket [5]. Such administration may not produce an adequate concentration at the action site as well as the problems associated with the side effects of high doses of an antibiotic. Alternatively to compensate such problems the local administration of the drug in a controlled release delivery system is selection of research. Appropriate materials for bio adhesion are mainly hydrogel forming polymers may be used for such hypothesis [6].

Antimicrobial agents are a relevant adjuvant treatment to mechanical debridement. The local or systemic administration of these agents removes or significantly decreases the putative periodontal pathogens in contaminated sites [7]. The efficacy of many antibiotics as periodontal therapies has been investigated. Metronidazole has been widely used for this purpose [8, 9] and it constitutes the main drug of choice for anaerobic infections. Metronidazole exhibits efficacy against anaerobic pathogens, low levels of microbial resistance, favourable pharmacodynamics and pharmacokinetics, few adverse effects, and low cost [10, 11]. The direct local administration of antimicrobial agents into the periodontal pocket minimizes systemic drug exposure.

2. Materials and Method

Materials:

Metronidazole and Minocycline HCl are the gift samples provided by SEIMENS Laboratories Gurgaon, India and Welcure Drugs and Pharmaceuticals, New Delhi, India respectively. Ethyl cellulose purchased from S.D. fine chem. Ltd, Mumbai. Polymethylmethacrylate, Hydroxy propyl cellulose are purchased from Sigma Aldrich, Steinheim, Germany. Hydroxy propyl methyl cellulose purchased from Titan biotech, Bhiwadi, Rajasthan, India.

Preparation of Periodontal Films:

The periodontal films are prepared by Solvent casting technique, in which the films are prepared by dissolving ethyl cellulose and in combination with other polymers such as, hydroxypropyl cellulose (HPC), hydroxypropylmethyl cellulose K4M (HPMC K4M), polymethyl methacrylate (PMMA1, 20,000) in chloroform and dichloromethane (1:1) mixture, dibutylphthalate (10% v/w of that of polymer) and glycerol (20%v/v) as plasticizers.

Consequently film of Eudragit L- 100 was prepared by dissolving Eudragit L-100 into 40:60 mixture of acetone: propane-2-ol and glycerol 10% v/w as plasticizer using magnetic stirrer in a closed beaker. Into this, MTZ and MINOCYCLINE HCl of required amount was added. After the complete mixing was done, 5 ml solution was poured over the inverted clean petridish covered, by the aluminium foil. After complete evaporation of solvent, cast films were obtained. The composition of films is given in Table 1.

Evaluation of Periodontal Films:

1. Thickness

Thickness of the film was measured by using calibrated micrometer screw gauge; with a least count of 0.01mm. The average of three determinations was made.

2. Folding Endurance

Folding endurance of the film was determined by repeatedly folding a small strip of film of 2x2 cm size at the same place till it ruptured at the site of folding. The experiment was carried out in triplicate.

3. Texture Analysis

Texture profile analysis (TPA) defines the mechanical parameters in terms of tensile strength, mucoadhesiveness, properties that will affect ease product application into and retention within, periodontal pocket. TPA also allows an estimation of extent of structural reformation following product application. Therefore in this regard, TPA is an applicable technique for the characterization of formulations designed for application to periodontal pocket.

4. Tensile Strength

Tensile strength of the films was noted on texture analyzer (TAXT plus Express) using a film strip of 4x 1 cm that was clamped on vertical clamps and the force required to break the film was measured. Test was carried out in tension mode with a pre test speed of 0.5mm/sec, applied force of 509.9 gm, return distance of 10 mm, contact time of 20 sec, trigger force of 5.0 gm.

5. Miucoadhesion

The mucoadhesion of formulations under investigation was determined using texture analyzer (TAXT Express). Periodontal films prepared were attached to the cylindrical plate and wetted with saliva to simulate the periodontal condition between gums and tooth. Test was carried out in tension mode with a return to start distance of 5 0cm, a pre test speed of 0.5 mm/sec and post test speed of 2 mm /sec, total distance travelled 30 mm/sec and trigger force of 5 gm .The probe was detached vertically at a constant upward speed of 1mm per second and the force required to detach the film was measured as the peak value in the force time plot.

6. Drug(S) Content:

Drug(s) content was determined by extracting a film of size $0.25cm^2$ in 0.1 N HC1. After complete solubilization of drugs, the solution was filtered and suitably diluted with phosphate buffer pH 7.4, absorbance recorded at 319nm, 273.8nm and 291.6nm and drug content was determined by simultaneous equation method as well as Q-Absorbance analysis.

7. *In Vitro* Drug(S) Release Studies

In vitro drug(s) release study was performed by placing a film of 0.25cm in a vial containing 5 ml of phosphate buffer pH 7.4. Sampling was done at 24 hours interval and medium was replaced with phosphate buffer pH 7.4 from 1st to 7th day. The samples were analysed spectrophotometrically and the drug(s) release was estimated by q1 equation method.

8. *In Vivo* Evaluation:

This study was approved by the local Ethics Committee for Animal Usage IAEC/PSIT/1273/ac/09. Five rabbits weighing 2-2.5 kg, was used in this study. All the procedures in the animals were performed under general anaesthesia. Periodontitis was induced according to Nociti Jr. et al. Cotton ligatures were placed subgingivally around the test teeth (upper and lower incisors and premolars). Each rabbit had 6 teeth ligated, resulting in a total of 30 teeth in which the formation of periodontal pockets was induced. During 30 days, the ligatures were left in place and the rabbits were fed a soft diet to promote plaque accumulation. After the ligation period, only pockets with probing depth of at least 4 mm were used for film application. The formulation used in this study consisted of PMMA 120,000 (Fl) 3 mg MTZ and 3.5 mg MINOCYCLINE HCl, DBA as plasticizer. The film was administered by means of periodontal probe in the test sites in a gentle probing manner, attempting to fill the full extent of the pocket.

8.1. Plaque Index

The measurement of the state of oral hygiene by plaque index is based on recording both soft debris and mineralized deposits on the following teeth. The scoring is shown in Table 2

Table 2: Plaque Index Calculations

Scores	Criteria
1	A film of plaque adhering to the free gingival margin and adjacent area of the tooth. The plaque may be seen in situ only after application of disclosing solution or by using the probe on the tooth surface.
2	Moderate accumulation of soft deposit a within the gingival pocket, or the tooth and gingival margin which can be seen with the naked eye.
3	Abundance of soft matter within the gingival pocket and/or on the tooth and gingival margin.

8.2. Gingival Index

The gingival index is based on the two characteristics signs of inflammation: swelling (oedema) redness. The scoring is shown in Table 3.

Table 3: Gingival Index Calculations

Scores	Criteria
0	Normal Gingiva
1	Mild Inflammation, slight change in color, slight oedema No bleeding on probing
2	Moderate Inflammation, Redness, Oedema Bleeding On probing
3	Severe Inflammation Marked redness and oedema Ulceration Tendency to spontaneous bleeding

8.3. Sulcus Bleeding Index (SBI)
The SBI is defined as follows: The scoring is shown in Table 4.

Table 4: Sulcus Bleeding Index (SBI) Calculation

Scores	Criteria
0	Gingiva of normal texture and color No bleeding
1	Gingiva apparently normal, bleeding on probing
2	Bleeding on probing, change in color, No oedema
3	Bleeding on probing, change in color, Slight oedema
4	Either Bleeding on probing, change in color, obvious oedema Or Bleeding on probing, obvious oedema
5	Bleeding on probing Spontaneous bleeding Change in color Marked oedema

8.5. Pocket Probing Depth:
A periodontal probe is an instrument in dentistry commonly used in the measurement of pocket depth. Probe has markings at 3mm and 8mm for measurement.

3. Results and Discussion

Evaluation of Periodontal Films

1. Thickness
The physiochemical evaluation data indicates that the thickness of periodontal film varied from 0.37mm to 0.41mm (Table 5), thus suggesting uniform thickness of the formulation designed, ensuring the reproducibility of the method.

2. Percentage Moisture Loss
For all the formulations, the percentage moisture loss varied between 0.54% to 12.5% (Table 5). Formulation F6 showed maximum of moisture loss owing to excessive water loss. Formulation Fl showed minimum percentage moisture loss because of the presence of hydrophobic polymethyl methacrylates. In general films casted with hydrophilic polymers exhibited greater moisture loss when compared to those formulated with hydrophobic polymers.

3. Folding Endurance
All the formulations exhibited a folding endurance of more than 200 suggesting the film to possess good elasticity as well as flexibility (Table 5). Maximum folding endurance was observed for F3 formulation consisting of ethyl cellulose and Eudragit L-100 containing dibutyl phthalate as plasticizer. Minimum folding endurance was of F9 formulation consisting of Eudragit L-100, probably be due to change in plasticizer i.e. glycerol instead of dibutyl pthalate.

4. Weight Variation
Standard deviation of the recorded weights of films was found to be of low order (Table 5) suggesting uniformity of periodontal films made by solvent evaporation method.

5. Tensile Strength
Tensile strength of films was found to be in the range of 37.281- 272.369 g/cm^2 (Table 5). The formulation Fl showed maximum tensile strength thus will be able to maintain its integrity during its handling and placement in periodontal pocket and will produce the presumed clinical effect. Formulation F7 consisting of hydroxy propyl methyl cellulose showed minimum tensile strength probably due to its hydrophilic the that can absorb moisture from the atmosphere thereby causing reduction in the strength.

6. Mucoadhesion Test
Textural analysis, adhesiveness is defined as the work required in removing the from the formulation following compression, a process which involves lage of the bonds between the probe and the formulation. Relationship has

been described between adhesion and bioadhesion for bioadhesive elations. The force required to detach each formulation are presented in Table. Formulation Fl required a proportionally greater vertical detachment force to the mucoadhesive bond than formulation F9.

7. Swelling Index

Formulation Fl showed higher swelling index and a direct relationship between the degree of polymer, hydration in vitro and the duration of bioadhesion in vivo is reported therefore, a good degree of in vitro swelling would predict satisfactory bioadhesion. The formulation F6 and F7 consists of hydroxyl propyl cellulose and hydroxy propyl methyl cellulose K4M are biodegradable polymers and dissolve readily in a medium.

8. Drug (S) Content Determination

For the spectrophotometric analysis performed in duplicate for all the samples, it was observed that percentage drug content of all the periodontal films ranged between 95.28% to 101.96% for both MTZ and MINOCYCLINE HCL as determined by simultaneous equation method and between 95.86% to 99.87% (Table 6) as determined by Q-absorbance ratio method.

9. Biodegradability Study

In particular, formulation F1 (mentioned in Table 7) maintained its integrity in all media tested. The presence of a-amylase increased the degradation of biodegradable polymers as because of its antiproteolytic activity. The biodegradative effect was more evident for all batches of films when soaked in phosphate buffer ph7.4, than when soaked in distilled water.

10. *In Vitro* Drug(S) Release

In vitro drug(s) release studies were carried out for formulations, Fl to F9 in, phosphate buffer ph 7.40. In vitro drug(s) release performed using phosphate buffer ph 7.40 showed an initial burst release which is expected to kill most of periodontal pathogen, followed by controlled release for about 7 days, (fig. 1 and fig ,Table 8&9) sufficient to inhibit growth of periodontal pathogens. Three formulations Fl, F2 and F7 were selected and study was done in triplicate and among these formulations F formulation was selected as an optimized formulation because it is able to maintain the sustained release of both MTZ as well as MINOCYCLINE HCL upto 85% to 93% for 7 days.

11. Antibacterial Activity

As the drug(s) concentration in the film was increased the zone of inhibition (Table 10) also increased. The microbiological studies at one end revealed that the drug released was able to inhibit the growth of microbes, as the regression value i.e. r^2 is 0.9848 it indicates that there is linearity in graph of zone of inhibition and diameter of film.

12. Stability Study

The result showed in Table 11 revealed that the optimized F1 formulation was stable when stored at room temperature ($25^o \pm 2^oC$), oven temperature (45 ± 2^oC) and in refrigerator ($5\text{-}8^oC$) for a period of 90 days as the drug(s) content was found determined to be greater than 95% percent till the end of three months.

13. *In Vivo* Evaluation

The optimized film Fl formulation along with scaling and root planing was effective in removing the local irritants, reducing gingival inflammation, reducing pocket depth, and increasing clinical attachment. It also controlled the localized infection and prevented new lesion formation. In the present study, a significant reduction in mean plaque index, gingival index, sulcus bleeding index, and probing pocket depth, and a significant gain in clinical attachment (Table 12) were observed.

Table 1: Formulation Design of Periodontal Films Prepared By Solvent Casting Technique

Formulation code	MTZ (mg)	Minocycline HCl (mg)	Plasticizer (W/v)	Ethyl cellulose (mg)	Hydroxy propyl cellulose (mg)	Hydroxy propyl methyl cellulose K4M (mg)	Eudragit-L 100 (mg)	PMMA 120,000 (mg)
F1	3	3.5	DBA	-	-	-	-	450
F2	3	3.5	DBA	400	-	-	-	250
F3	3	3.5	DBA	400	-	-	125	-
F4	3	3.5	DBA	400	100	-	-	-
F5	3	3.5	DBA	400	-	100	-	-
F6	3	3.5	DBA	-	-	450	-	-
F7	3	3.5	DBA	-	450	-	-	-
F8	3	3.5	DBA	450	-	-	-	-
F9	3	3.5	Glycerol (20%v/v)	-	-	-	250	-

Table 5: Physical Properties of Periodontal Film Prepared By Solvent Casting Technique

Film composition	Thickness (mm)	Folding endurance	Weight variation	Swelling index	Mucoadhesion (g/cm^2)	Tensile strength (g/cm^2)	Percent moisture loss
F1	0.38+0.02	300+2.05	13.9+0.124	0.20	95.756	272.369	0.54+0.09
F2	0.37+0.03	226+3.09	10.0+0.081	0.067	46.560	108.128	2.78+0.44
F3	0.41+0.08	359+3.39	10.0+0.081	0.102	59.811	59.811	0.27+0.16
F4	0.40+0.01	222+6.16	14.1+0.04	0.08	64.862	54.101	6.00+0.10
F5	0.37+0.01	306+3.74	6+0.081	0.22	21.816	126.064	2.22+0.009
F6	0.40+0.02	339+4.10	12.1+0.047	Soluble	5.051	37.775	12.5+0.55
F7	0.41+0.01	256+4.32	11.1+0.081	solube	26.208	37.281	0.63+0.13
F8	0.39+0.03	240+4.08	13.3+0.047	0.064	28.624	34.042	1.40+0.23
F9	0.38+0.02	201+4.98	13.3+0.047	0.032	76.314	75.916	2.34+0.09

Table 6: Drug(S) Content Determined by Simultaneous Equation Method And Q-Absorbance Ratio Method.

Periodontal Films	Simultaneous Equation Method	
	MTZ	MINOCYCLINE HCl
F1	96.28±1.01	98.36.96±1.46
F2	98.16±2.22	98.21±2.03
F3	98.65±2.44	99.44±0.575
F4	98.15±3.25	98.27±0.132
F5	97.33±3.45	99.59±0.315
F6	96.74±2.47	97.83±0.37
F7	99.08±1.01	99.88±0.74
F8	98.45±2.1	99.26±0.16
F9	95.25±3.7	99.87±0.76

Table 7: Film Degradation Study In 0.9% W/V Nacl Solution, Distilled Water And Phosphate Buffer Ph 7.40 Each Containing 0.05% W/V A-Amylase Enzyme.

S.No	Periodontal films	Film Degradation Study In Three Different Medium For 60 Days																	
		MEDIUM I (0.9% w/v NaCl solution and 0.05% w/v a-amylase enzyme)						MEDIUM II (Distilled water containing 0.05% w/v a-amylase enzyme)						MEDIUM III (PHosphate buffer ph 7.4 containing 0.05% w/v a-amylase enzyme)					
		10^{th} day	20^{th} day	30^{th} day	40^{th} day	50^{th} day	60^{th} day	10^{th} day	20^{th} day	30^{th} day	40^{th} day	50^{th} day	60^{th} day	10^{th} day	20^{th} day	30^{th} day	40^{th} day	50^{th} day	60^{th} day
1.	F1	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
2.	F2	N	S	S	S	S	S	N	S	S	S	S	S	N	S	S	S	S	S
3.	F3	N	N]	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
4.	F4	N	N	S	S	S	S	N	N	N	S	S	S	N	N	S	S	S	S
5.	F5	N	N	S	S	S	S	N	N	N	T	T	T	N	N	T	T	T	T
6.	F6	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
7.	F7	T	T	T	T	T	T	N	T	T	T	T	T	N	T	T	T	T	T
8.	F8	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
9.	F9	S	T	T	T	T	T	S	T	T	T	T	T	S	T	T	T	T	T

N - Not degraded
T- Totally degraded
S - Slightly degraded

Table 8: Percent Drug(S) Release Of Periodontal Films In Phosphate Buffer Ph 7.40 Formulations (F2-F5).

Days	F2		F3		F4		F5	
	MTZ	MINOCYCLINE HCl	MTZ	MINOCYCLINE HCl	MTZ	MINOCYCLINE HCl	MTZ	MINOCYCLINE HCl
0	0	0	0	0	0	0	0	0
1	71.33	81.42	71.12	60.66	60.66	50.20	70.63	71.35
2	72.32	78.52	72.65	75.14	61.49	57.04	72.67	73.38
3	72.37	77.24	73.28	75.63	62.63	58.54	73.21	74.54
4	73.66	77.84	74.44	76.17	63.34	59.36	73.51	75.24
5	73.58	77.63	75.34	76.52	64.59	60.21	73.68	75.36
6	74.57	77.37	75.84	76.23	65.84	61.36	74.89	76.12
7	75.52	77.91	76.81	76.66	66.84	63.32	74.56	76.52

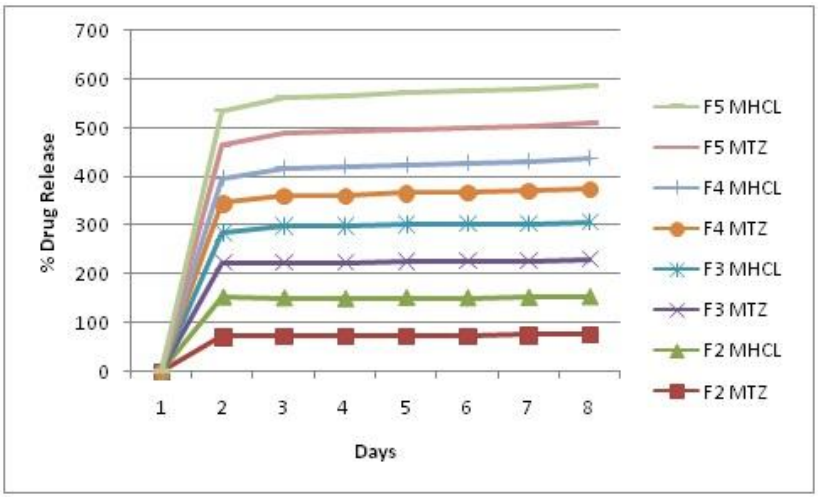

Figure 1: Percent Drug(S) Release Of Periodontal Films In Phosphate Buffer Ph 7.40 Formulations (F2-F5)

Table 9: Percent Drug (S) Release of Mean of Selected Formulations (N=3)

Days	F1		F2		F7	
	MTZ	MINOCYCLINE HCl	MTZ	MINOCYCLINE HCl	MTZ	MINOCYCLINE HCl
0	0±0	0±0	0±0	0±0	0±0	0±0
1	82.95±1.90	91.56±0.55	71.81±0.70	81.92±1.29	68.79±1.72	89.58±2.13
2	84.55±2.22	93.77±2.34	72.55±0.98	78.83±1.20	69.92±2.52	92.29±1.39
3	84.32±0.65	93.28±3.07	72.13±2.20	77.84±2.79	68.38±3.01	92.18±3.00
4	84.03±0.89	93.56±0.67	73.29±0.83	77.11±3.33	72.78.±2.99	92.58±2.67
5	86.06±1.96	94.71±0.53	73.17±1.52	77.03±0.98	73.38±0.39	92.19±0.77
6	86.64±0.81	94.92±0.78	74.68±1.39	77.89±3.65	73.70±3.52	93.97±8.88
7	87.44±0.18	93.35±0.58	75.00±2.98	77.71±2.98	73.18±2.80	93.64±1.87

Table 10: Showing Effect of Increasing Drug (S) Concentration on Zone of Inhibition

Formulation	Surface area (cm^2)	Diameter of film (mm)	Zone of inhibition (cm) (mean±s.d.)
F1	0.25	5.64	6.7±0.14
F1	0.41	7.22	7.4±0.10
F1	0.83	10.3	8.9±0.07
F1	1.16	12.18	11.25±0.09

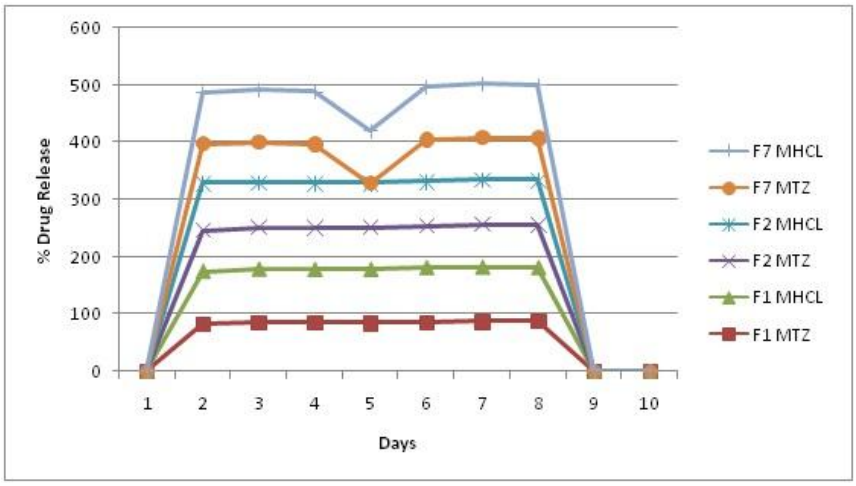

Figure 2: Percent Drug (S) Release of Mean of Selected Formulations (N=3)

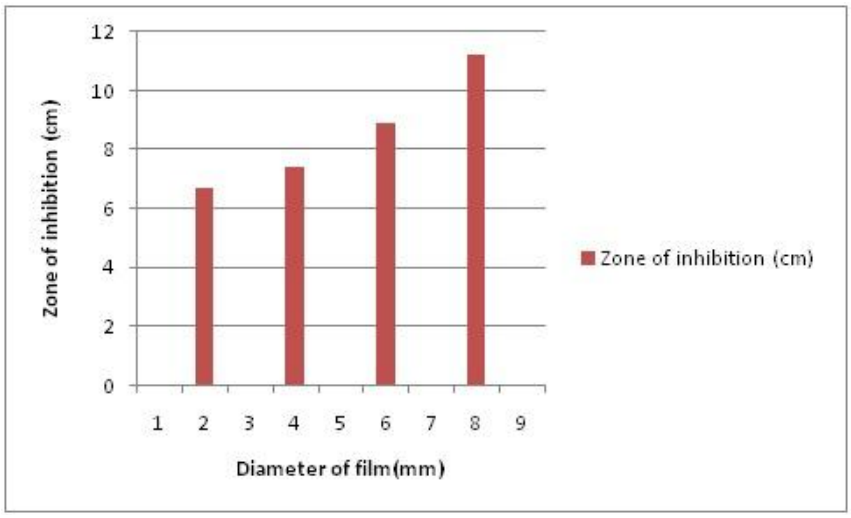

Figure 3: Graph Between Diameter Film And Zone of Inhibition

Table 11: Stability Study of Periodontal Films

S.No.	Time (months)	% Drug(s) content					
		Room temperature ($25^{o}\pm2^{o}C$)		Oven temperature ($45\pm2^{o}C$)		Refrigerator ($5\text{-}8^{o}C$)	
		MTZ	MINOCYCLINE HCl	MTZ	MINOCYCLINE HCl	MTZ	MINOCYCLINE HCl
1	1	98.51	98.05	96.83	97.25	99.30	98.55
2	2	97.83	97.05	96.83	97.55	98.58	98.58
3	3	99.01	98.25	86.72	97.52	97.81	98.02
Appearance		Smooth, yellow		Smooth, yellow		Smooth, yellow	

Table 12: *In Vivo* Studies of Periodontal Films

Time (Days)	Plaque Index	Gingival Index	Sulcus Bleeding Index	Pocket Probing Depth (mm)
7	3	3	4	6
14	2	2	2	5
21	1	1	1	2
28	0	0	0	0

4. Conclusion

A bioadhesive periodontal film for simultaneous extended delivery of Metronidazole and Minocycline HCl into periodontal pocket was formulated. The film (F1 formulation) displayed dose dependent antibacterial activity and was found to be stable. The approach provides an opportunity and potential for development of periodontal film containing both Metronidazole and Minocycline HCl for extended release. In the present study, a significant reduction in mean plaque index, gingival index, sulcus bleeding index, and probing pocket depth, and a significant gain in clinical attachment, were observed

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