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Development, in-vitro characterization and ex-vivo permeation study of metronidazole loaded mucoadhesive ethosomal gel for the local treatment of oral cavity infection

Jitendra Debata
Associate Professor, Guru Nanak Institutions Technical Campus, School of Pharmacy, Ibrahimpatnam Hyderabad, Telangana, Pin:501506

V. Ravi Kumar
Associate Professor, Guru Nanak Institutions Technical Campus, School of Pharmacy, Ibrahimpatnam Hyderabad, Telangana, Pin:501506

Dibyalochan Mohanty
Associate Professor, Department of Pharmaceutics, School of Pharmacy, Anurag University, Hyderabad, Telangana, Venkatapur, Pin:500088

B. Nirosha
Assistant Professor, Department of Pharmaceutics, School of Pharmacy, Anurag University, Hyderabad, Telangana, Venkatapur, Pin:500088

Om Prakash Panda
Assistant Professor, Centurion University of Technology and Management, Odisha, India

Ch Praveen Kumar
Associate Professor, Swathi College of Pharmacy, Nellore, Andhra Pradesh,Pin:524320

Vasudha Bakshi
Professor & Dean, School of Pharmacy, Anurag University, Hyderabad, Telangana, Venkatapur, Pin:500088

***Abstract*---**The present study deals with the formulation, characterization of Metronidazole loaded ethosomal gel for treating oral infection. The ethosomal system of Metronidazole comprises of 2-5% phospholipids, 20-50% ethanol, 10% of propylene glycol, 10% of cholesterol and 10% v/v aqueous phase. Cold method was applied to develop ethosome. The prepared Metronidazole loaded ethosome was evaluated for vesicle size, entrapment efficiency,The Optimized

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Ethosome formulation was converted in to ethosomal gel. The gel further evaluated for pH, Drug content, mucoadhesive strength and ex-vivo permeation study. The vesicle size of ethosome formulation MEF8 showed the lowest value of 324±1.32 µm. Formulation MEF8 showed the maximum entrapment efficiency of 86.11±0.11%. The mucoadhesive gel MEG8 formulated from optimized ethosome showed the maximum drug release 99.35% in 12 hr and ex-vivo oral mucosa permeation for MEG8 was found to be 1200µg/ml at 30minutes as compared to the pure drug.

***Keywords*---**Ethosome, Entrapment Efficiency, Metronidazole, Ex-vivo Permeation.

Introduction

The type of bacteria found within odontogenic infections are part of the microbiota of the oral cavity. These infections are frequently polymicrobial and their invasiveness may be determined by the specific combinations present as specific bacteria vary in their pathogenicity. More than 700 bacterial species have been identified in the oral cavity[1] .the human oral microbiome consists of the microorganisms present within the oral cavity and its adjacent structures extending to the distal third of the esophagus.[2] These structures provide distinct microbial habitats that include the teeth, gingiva, tongue, hard and soft palates, cheeks, and lips. Contiguous structures, such as the tonsils, pharynx, Eustachian tube, middle ear, trachea, lungs, nasal cavity, and sinuses, also provide a niche for various microorganisms.[3]

Metronidazole (MET) is regarded as a extremely effective antimicrobial agent in treating adult periodontic ailment [4]. MET, which is (1-(β-hydroxyethyl)-2-methyl-5-nitroimidazole), a synthetic nitroimidazole derivative, is used as an antibacterial agent with added antiparasitic properties.[5] It is generally very well tolerated, and it has a wide therapeutic index. MET possesses poor water solubility and hydrophobicity. MET's solubility in water was reported as 10.5 mg/mL at 25 ◦C owing to a low log P value (−0.18)[6], MET is classified as a hydrophilic drug. It is available in a variety of dosage forms, such as capsules, tablets suppository preparations and topical formulations, which are used for the treatment of different infections[7]. In this investigation metronidazole ethosmal gel was formulated and evaluated for gel strength, ex vivo oral mucosa permeation and antimicrobial activity

Materials & Methods

Metronidazole was obtained as a gift sample from MSN Lab Hyderabad (India). Soya lecithin was purchased from Hi-Media labs. Carbopol 934 was purchased from Sigma Chemicals (USA). All other chemicals used in this study were of analytical grade

Preparation of Metronidazole Ethosome (Cold Method)

Preparation Metronidazole ethosomes was followed by method suggested by Touitou et al., The ethosomal system of Metronidazole comprises of 5-15% phospholipids, 5-10% ethanol, 5% of propylene glycol, 10% of cholesterol and aqueous phase to 10% v/v. Metronidazole , phospholipid and other lipid materials were dissolved in ethanol in a covered vessel at room temperature by vigorous stirring[8,9]. Propylene glycol was added to water. This mixture was heated to 30^0C in a separate vessel and was added to the former mixture drop wise in the centre of the vessel, while stirring[10]. The mixture was kept for stirring for 30 mins at 700 rpm in a covered vessel .the ethosomal suspension was subjected to probe sonicator to reduce the vesicle size .finally the formulation was stored under refrigeration.

Table 1: Formulation table for Metronidazole loaded ethosome

Formulation	Soya lecithin %	Ethanol (%)	Propylene glycol (%)	Drug (mg)	Cholesterol (%)	Water(ml)
MEF1	15	5	5	65	10	10
MEF 2	10	5	5	65	10	10
MEF 3	5	5	5	65	10	10
MEF 4	15	7.5	5	65	10	10
MEF 5	10	7.5	5	65	10	10
MEF 6	5	7.5	5	65	10	10
MEF 7	15	10	5	65	10	10
MEF 8	10	10	5	65	10	10
MEF 9	5	10	5	65	10	10

Characterization of Ethosomes
Vesicle Size and Shape Analysis

Microscopic analysis was performed to determine the average size of ethosomes. A sample of ethosomes was suitably diluted with distilled water in order to observe individual vesicle and a drop of diluted suspension was put on a glass slide covered with cover slip and examined under microscope (magnification 15x 45x). The diameters of 150 vesicles were determined randomly using calibrated eyepiece micrometer with stage micrometer. The average diameter was calculated using the formula.

Average diameter = Nd/N N = number of vesicle d = diameter of vesicles

Entrapment Efficiency

The entrapment efficiency of ethosome was determined by ultracentrifugation[11,12]. 10ml of each sample was vortexed for 2 cycles of 5min with 2 minutes rest between the cycles. 1.5ml of each vortexed sample and fresh untreated ethosomal formulations were taken into different centrifugal tubes. These samples were centrifuged at 20,000rpm for 3 hours. The supernatant layer was separated, diluted with water and drug concentration was determined at 277nm in both

vortexed and un vortexed samples. The entrapment efficiency was calculated as follows

Entrapment efficiency = [(T-C)/T]x 100

T' is total amount of drug that detected from supernatant of vortexed
'C' is the amount of drug un entrapped and detected from supernatant of un vortexed

Preparation of Metronidazole Gel

Nine Batches of Metronidazole gel **MEG1 to MEG9** were formulated. the best achieved ethosomal suspension was incorporated into Carbopol 934 (1% to 9%)gel. 0.2% methyl paraben,1% of (v/v) triethanolamine was added with continues stirring to form transparent alkaline gel. Water also added with continuous stirring until homogenous formulation were achieved.

Formulation	Ethosomal suspension(ml)	Carbopol 934(% v/v)	Methyl paraben	triethanolamine
MEG1	10	1%	0.2%	1%
MEG2	10	2%	0.2%	1%
MEG3	10	3%	0.2%	1%
MEG4	10	4%	0.2%	1%
MEG5	10	5%	0.2%	1%
MEG6	10	6%	0.2%	1%
MEG7	10	7%	0.2%	1%
MEG8	10	8%	0.2%	1%
MEG9	10	9%	0.2%	1%

Characterization of Gel

pH: Solution of 1gm of gel dissolved in 30ml of distilled water (pH-7) was prepared. The pH of the ethosomal gel was determined by using digital pH meter.
Drug Content and Content Uniformity: 1gm of gel was dissolved in a100ml of phosphate buffer pH 7.4 for 48 hours with constant stirring using magnetic stirrer. Solution was filtered and observed under UV spectrophotometer at lamda max 277nm. The measurements were made in triplicate.
Viscosity The viscosity was measured by Brookfield viscometer using T spindle S-63 at 100 rpm. The temperature was maintained 30º.

Scanning Electron Microscope (SEM)

Scanning electron microscopy was conducted to characterize the surface morphology of ethosomal formulation, for which a drop of ethosomal formulation was mounted on clear glass stub, air-dried and coated with Polaron E 5100 Sputter coater (Polaron, UK) and visualized under SEM (JEOLJSM-6510, Japan[13]

In- Vitro diffusion studies

The in vitro diffusion of Metronidazole from ethosmal gel formulation was studied using Franz diffusion cell specially designed in our laboratory according to the literature. The effective permeation area of the diffusion cell and receptor cell volume was 2.4cm and 200ml respectively[14]. The temperature was maintained at 37± 0.5°C. The receptor compartment contained 200ml of pH 7.4 buffer and was constantly stirred by magnetic stirrer at 100rpm. Prepared dialysis was mounted between the donor and the receptor compartments. Ethosomal gel formulation was applied to the dialysis membrane and the content of diffusion cell was kept under constant stirring then 5ml of samples were withdrawn from receptor compartment of diffusion cell at predetermined time intervals and analysed by the UV-spectrometric method at 277nm after suitable dilution. The receptor phase was immediately replenished with equal volume of fresh pH 6.8 buffer. Triplicate experiments were conducted for drug release studies up to 12hr

Ex-vivo permeation studies

Ex-vivo permeation studies were carried out through porcine oral mucosa using modified Franz diffusion cell . This system consists of a Donor chamber and receptor cell. Jacket and sampling port[15] . The buffer was warmed with the in built heater and then assembly was set thermos statically at 37^{o}c. A Teflon coated mini magnetic bead was placed in the receptor compartment for agitating the contained vehicle at 50rpm. The receptor compartment was filled with vehicle containing phosphate buffer pH6.8 receptor fluid was sonicated to remove the air bubbles and equilibrated at 37^{o}C before placing the receptor component. The mucosa was mounted between the Donor and receptor compartment. The receptor component was filled with 5ml of isotonic phosphate buffer of pH6.8 buffer magnetic bead was placed and rotations were 100rpm was maintained. In an interval of every 5min 1ml sample was withdrawn and replaced with fresh phosphate buffer medium at suitable time intervals of 5,10,15,20,25,30min respectively. The samples were analysed in UV-Spectrophotometer.

Antimicrobial activity

Antimicrobial study was carried out to check the antimicrobial efficiency of Metronidazole ethosomal gel. The test organisms used were Candida albicans; the growth medium used was nutrient agar. The cup-plate method was used to carry out antimicrobial study. The method is based on the principle of diffusion of drug from vertical cup through solidified agar layer in Petri plate. Sterile solution of Metronidazole (on the market) gel was used as a standard. The standard solution and the developed formulations (test solution) were taken into separate cups bored into sterile nutrient agar previously seeded with Candida albicans organisms. The gels were allowed to diffuse for 2 h and then the plates were incubated for 24 h at 37°C. The zone of inhibition (ZOI) was compared with that of the standard.

Results & Discussion

FTIR Study

IR spectra of pure drug and ethosomal gel was compared and checked for any shifting functional peaks .From the spectral analysis it is clear that there is no interaction between the selected lipid, drug and mixtures. Hence the selected lipid was found to be compatible in entrapping metronidazole without any mutual interactions (Figure 1&2)

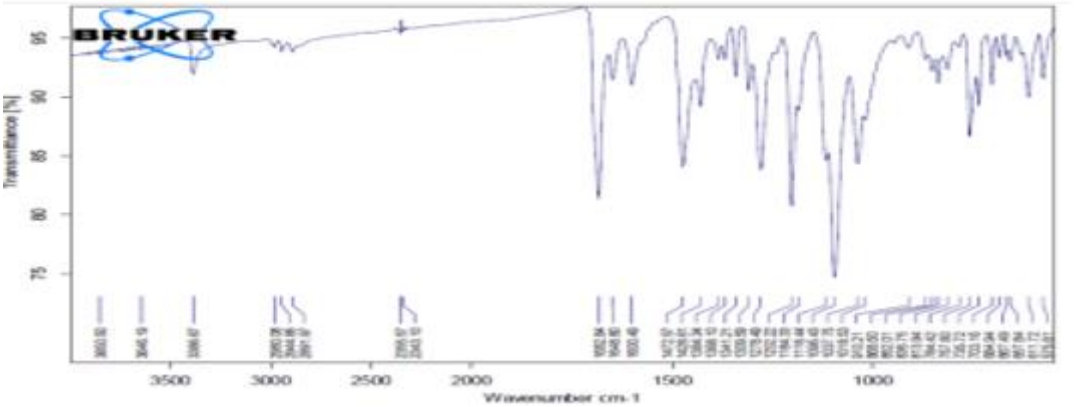

Figure:1 FTIR of pure Metronidazole

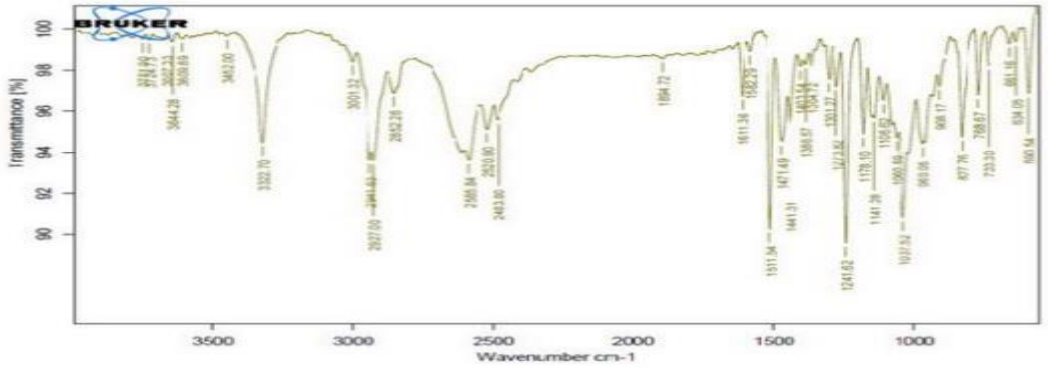

Figure:2 FTIR of optimized Metronidazole ethosomal gel

Vesicle Size and Entrapment Efficiency of Metronidazole ethosome

The vesicle size analysis of Metronidazole ethosome formulations was done by photon correlation spectroscopy (PCS), using a computerized inspection system (Zetasizer, HAS 3000; Malvern Instruments, Malvern, United Kingdom) at 25 ± 1◦C and at a scattering angle of 90◦.Samples were appropriately diluted with double-distilled water previously filtered with 0.45µm membrane filters before analysis. The vesicle size obtained in a size range between 324±1.32 µm to 681±1.56 µm(Table:2& Figure 3a) which can be easily pass through oral mucosa
For determination of entrapment efficiency the unentrapped drug in Ethosome formulations was separated by the use of the centrifugation method. A known

dilution of the ethosome was prepared with double distilled water and was centrifuged at 14,000 rpm (Cooling Centrifuge, C24, REMI Instruments Ltd., Mumbai, India) for 40 min at 10◦C. The entrapment efficiency obtained in the range of 73.12±0.12 % to 86.11±0.11%.(Table:2& Figure 3b)

Table: 2 Vesicle size and Entrapment efficiency of metronidazole loaded ethosome

Formulation code	Vesicle size (µm)	Entrapment Efficiency (%)
MEF1	681±1.561	73.12±0.12
MEF 2	539±1.41	78.31±0.32
MEF 3	612±2.21	77.31±0.22
MEF 4	532±1.12	76.31±0.25
MEF 5	527±1.11	77.81±0.12
MEF 6	548±1.02	79.41±0.21
MEF 7	395±1.25	82.61±0.24
MEF 8	324±1.32	86.11±0.11
MEF 9	423±1.32	80.61±0.13

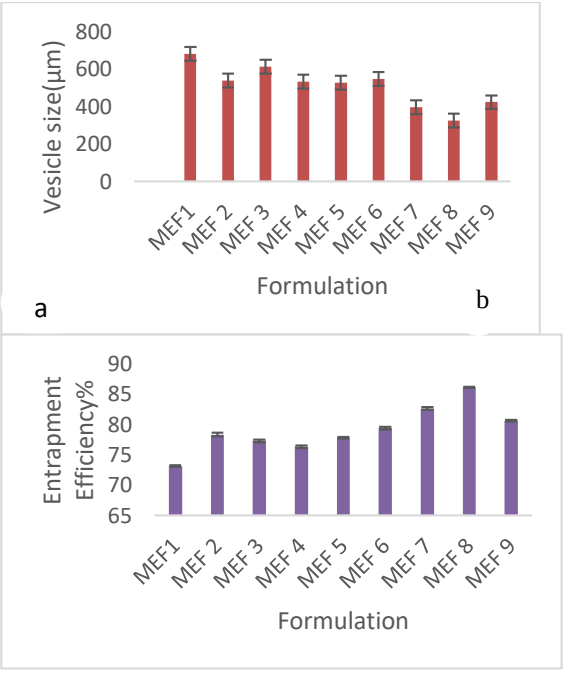

Figure3 : a)Vesicle size b) Entrapment Efficiency%

Characterization of Metronidazole ethosomal gel

Ethosomal mucoadhesive gel was characterized for drug content, pH, mucoadhesive strength & viscosity. Drug content for all gel formulation found to be more than 90%, pH for all formulation found in a range between 6.07 to 6.81. Mucoadhesive strength% found to be between 82.11% to 96.17% .The viscosity is found in between 276 to 292 poise.(Table:3& Figure 4)

Table: 3 Physical evaluation of Ethosomal mucoadhesive gel

Batch Code	pH	Drug content (%)	Mucoadhesive strength (%)	Viscosity (poise)
MEG1	6.81	95.14	82.11	290
MEG2	6.21	97.32	91.04	278
MEG3	6.71	92.34	93.11	276
MEG4	6.07	91.02	92.11	287
MEG5	6.42	98.25	94.03	281
MEG6	6.24	96.36	89.04	288
MEG7	6.78	92.45	95.17	283
MEG8	6.81	99.33	96.17	292
MEG9	6.79	93.47	95.34	287

MEG=Metronidazole Ethosomal Gel

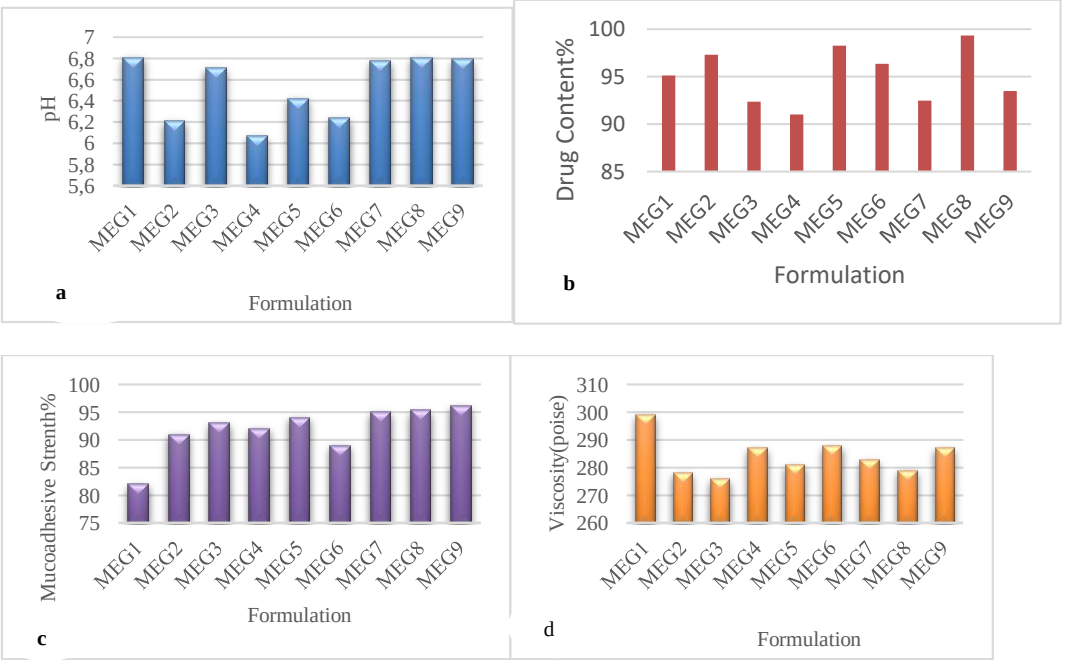

Figure no: 4 a) pH of mucoadhesive gel b) Drug content of mucoadhesive gel c) Mucoadhesive strength % d)Viscosity of mucoadhesive gel

In-vitro drug diffusion Study

The in vitro-drug diffusion studies were carried out by Franz diffusion cell.The maximum drug release was observed for the Gel formulation MEG8 which is 99.35% in 12 hour, the drug release profile for all gel formulation was described in figure no 5 & Table no 4

Table : 4 In vitro Release Study of Metronidazole ethosomal gel in 6.8 pH

Time (hr)	FORMULATION % of Drug Release								
	MEG1	MEG2	MEG3	MEG4	MEG5	MEG6	MEG7	MEG8	MEG9
0	0	0	0	0	0	0	0	0	0
0.5	8.71	8.11	11.98	5.02	10.01	23.11	9.01	5.12	12.98
1	22.34	21.34	32.34	11.34	22.34	39.77	12.34	12.14	32.14
2	38.54	44.14	43.04	33.54	33.54	54.11	33.54	41.12	53.04
3	49.71	51.01	53.11	73.71	43.71	70.21	43.71	64.21	68.11
4	77.14	62.33	78.24	82.11	72.14	89.11	72.14	71.26	77.24
5	89.13	79.13	88.13	91.23	88.13	98.16	88.13	78.13	83.13
6	99.11	84.11	92.11	99.72	90.11	-	99.11	85.03	95.11
7	-	89.31	98.11	-	99.13	-	-	89.11	98.11
8	-	90.11		-		-		82.99	
12								99.35	

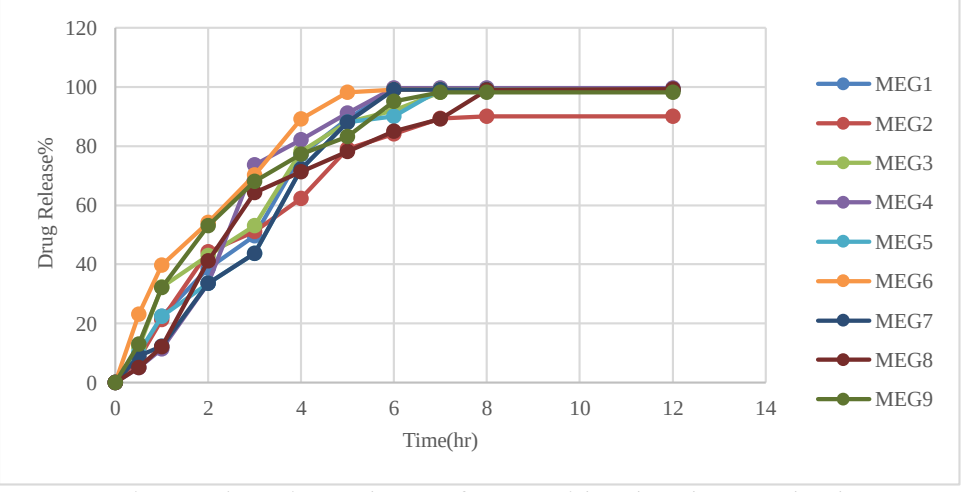

Figure: 5 In vitro release of Metronidazole ethosomal gel

Ex-vivo Permeation Study

In ex-vivo permeation study pure drug was compared with optimized gel formulation MEG8, Pure drug showed a permeation of 798µg/ml at 30minutes whereas optimized gel formulation MEG8 showed a permeation of 1200µg/ml at 30minutes(Figure:6)

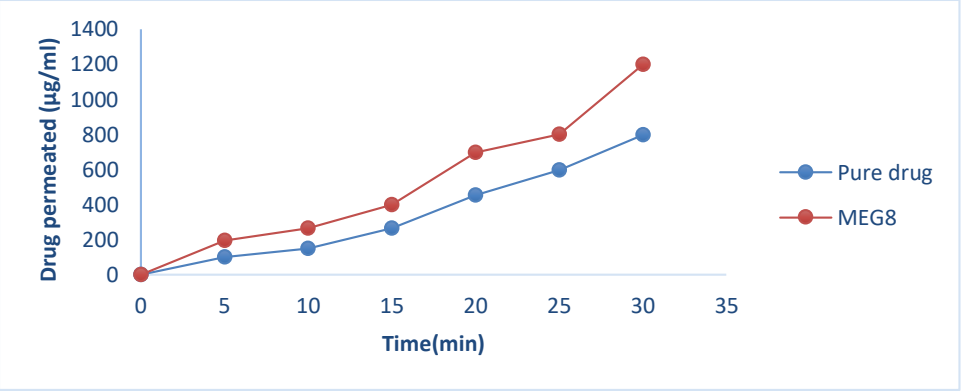

Figure: 6 Ex-vivo permeation study of Metronidazole ethosomal gel

Surface Morphology Study

Best ethosomal formulation MEG8 under SEM showed spherical shape and no aggregation was found in the SEM Study (Figure:7)

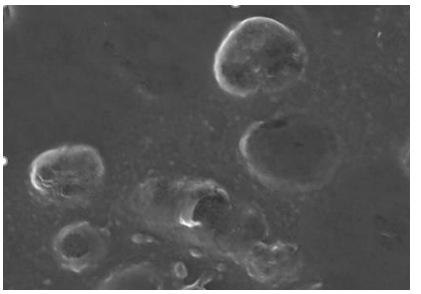

Figure : 7 SEM image of Metronidazole ethosomal gel

Antimicrobial Study of Metronidazole ethosomal gel

The antimicrobial study was carried out by agar cup plate method as qualitative assessment to compare antibacterial efficacy of developed MEG 8 with marketed gel Marketed metronidazole gel preparation shows a zone of inhibition of 45 mm for Candida Albicans compared to zone of inhibition of 43 mm obtained in MEG 8 in 24 h. As it can be seen, there is no observable difference between the zone of inhibition of free Metronidazole Ethosomal gel Metronidazole. This means that, loading of Metronidazole on ethosome does not lead to an enhancement in the antimicrobial function of Metronidazole. In other words, ethosome has no antimicrobial activity itself. Finally it concluded that the developed MEG 8 are equivalent to marketed Metronidazole gel in antibacterial action.

Table : 5 Antimicrobial activity of Metronidazole Ethosomal Gel (MEG-8) & Marketed Metronidazole Ethosomal Gel

Sl no	Formulation	Microorganism	ZOI(mm)
`1	Metronidazole Ethosomal Gel (MEG-8)	Candida Albicans	43
2	Marketed Metronidazole Ethosomal Gel	Candida Albicans	45

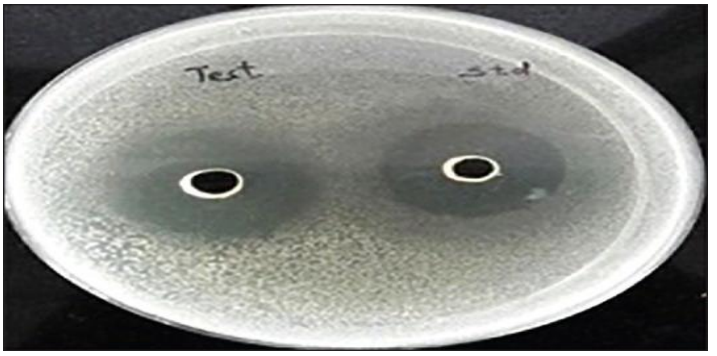

Figure: 8 Agar plates incubated for antimicrobial activity

Conclusion

In the present study, Metronidazole entrapped ethosomal gel for oral mucosa was prepared by using various concentrations of Soya lecithin and ethanol. The prepared formulation showed good entrapment efficiency, particle size and drug release. The antimicrobial result advocates the superiority of metronidazole loaded ethosomal formulation over the Marketed formulation. In conclusion, it can be suggested that ethosomes could be superior drug carrier for oral mucosa application of Metronidazole in the treatment of oral infection.

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