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DESIGN, DEVELOPMENT AND OPTIMIZATION OF NOVEL NANOVESICLE LOADED SUBGLOSSAL FILMS FOR ENHANCED SYSTEMIC AVAILABILITY OF TACROLIMUS THROUGH SUBLINGUAL ROUTE

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Ethosomes, Tacrolimus, Organ transplantation, Sublingual films, Pharmacokinetics

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ABSTRACT: Tacrolimus being the drug of choice in prevention of transplanted organ from rejection suffers from low oral bioavailability and dose-dependent side effects. An attempt was made to formulate tacrolimus into subglossal (sublingual) fast dissolving films containing ethosomes using ethanol and soyalecithin to enhance systemic availability of drug with controlled delivery of drug. Ethosomes were prepared by modified mechanical dispersion method and were optimized by central composite design to arrive for best drug-loaded ethosome dispersion. Optimized formulations were characterized for vesicle size, Deformability index and entrapment efficiency. Subglossal films were prepared for best and optimized dispersion using HPMC K15M and PEG-400 as film-forming polymer and plasticizer respectively. Formulated fast dissolving subglossal films were optimized by Box benhkendesign and characterized for physical characteristics like appearance, pH, thickness, disintegration time, *in-vitro* release, Histopathology studies, stability and *in-vivo* pharmacokinetics in rats. Higher drug plasma levels were shown by optimized subglossal film when compared with that of oral pengraf capsules. These results suggests the potential of nanovesicle fast dissolving films as effective drug carriers in sustaining therapeutic concentrations of tacrolimus for extended period in organ transplanted patients.

INTRODUCTION: Solid organ transplantation has evolved from merely being a clinical experiment into new era of medicine by giving quality life to many patients when compared with non-transplantation management strategies of both enduring and acute end stage organ failures [1].

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Histocompatibility antigens of genetically disparate tissues leading to rejection of an organ are encoded on more than 40 loci, but the loci responsible for the most vigorous allograft rejection reactions were found to be present on the major histocompatibility complex (MHC).

Rejection can be hyperacute or acute where the transplanted tissue may be rejected within minutes to hours or in the first 6 months after transplantation. Chronic rejection may be developed in months to years after acute rejection episodes have subsided [2-4]. Immunosuppressive agents play a pivotal role in moderating the

immune response and in minimizing the loss of the allograft and in increasing the life span of transplanted patients. The most commonly used immunosuppressants are calcineurin inhibitors (cyclosporine and tacrolimus) and their newer analogues (sirolimus and everolimus), inhibitors of nucleotide synthesis (e.g., azathioprine, mycophenolatemofetil, leflunomide), cytostatic agents affecting T-cell and B-cell division (e.g., methotrexate, mercaptopurine and gemcitabine), phosphodiesterase-4 (PDE4) inhibitors (mostly used as anti-inflammatory agents in COPD and autoimmune diseases), antibodies and other biological approaches [5, 6].

Tacrolimus being macrolide antibiotic, product of fermentation of Streptomyces is most widely used immunosuppressive drug for preventing solid-organ transplant rejection. It prevents the production of Interleukin-2 by inhibiting calcineurin via binding to FKBP12 protein. Tacrolimus is approximately 99% protein bound, widely distributes into most tissues including the lungs, spleen, heart, kidney, pancreas, brain, muscle and liver [7].

Tacrolimus has a narrow therapeutic window with half-life ranging from 11.7 to 34.8 h [8] and substrate of P-glycoprotein (P-gp) and cytochrome P450 3A4 (CYP3A4). Tacrolimus is poorly water soluble (4-12 μg/mL), with low oral bioavailability showing high intra and inter-subject variability with a mean bioavailability of 17-22% [9, 10]. Formulating tacrolimus into the nanovesicular dosage forms may correct some of these shortcomings, resulting in the enhancement of tacrolimus bioavailability. Nanovesicular dosage found to have substantial advantages over conventional dosage forms in delivering high molecular weight drugs in controlled manner due to their reservoir effect and high flexibility characteristics.

Ethosomes are lipid vesicles containing phospholipid and ethanol which acts by destabilizing the lipid bilayer and hence increasing the flexibility and penetration [11]. Due to the flexibility of their structural characteristics like composition, fluidity and size these lipid vesicles can be used for delivery of a wide variety of drugs for drug targeting controlled release and permeation enhancement. Fast dissolving films have gained stupendous benchmark in the pharmaceutical industry due to the improved patient compliance, accurate dosing and rapid onset of action with their convenient handling and administration [12-15]. For systemic drug delivery, fast dissolving films can be used *via* sublingual routedue to the high vascularity and permeability of this region, which allows for rapid absorption and quick action of the incorporated drug. The sublingual route is expected to enhance the drug bioavailability by avoidance of first-pass hepatic metabolism with rapid absorption of the drug-loaded vesicular carriers by maintaining therapeutic concentrations of the proposed drug for prolonged time period with minimal adverse effects related to oral route [16-21].

Administration of drugs sublingually found to be beneficial as absorption is found to be 3 to 10 times greater than oral route. It has been reported that approximately only 50% of the tacrolimus oral dose was needed to obtain therapeutic trough concentrations when converted to sublingual administration and since immunosuppressant drugs are given as maintenance therapy i.e on chronic basis most of the adverse effects which are dose dependant like nephrotoxicity can be reduced. As of now tacrolimus is available only in the form of tablets & capsules commercially but existing marketed products exhibits low oral bioavailability due to extensive presystemic metabolism and mean oral bioavailability was found to be only 21%.

Although i.v product is available, it can be toxic to the kidneys, found to be associated with allergic reactions and moreover i.v route suffers from patient non-compliance. Till now no study has been reported in open literature on the use of nanovesicular carrier for the delivery of the tacrolimus through sublingual route [22]. Hence, in the present study, an attempt has been made to design, optimize and characterize subglossal fast dissolving filmscontaining tacrolimus loaded ethosomes through sublingual route which may help in maintaining therapeutic concentrations of the tacrolimus for prolonged time period with controlled delivery by avoidance of first-pass hepatic metabolism.

MATERIALS: Tacrolimus was supplied from Dr Reddy's Labarotories, Soya lecithin was provided

by VAV Lipids, Mumbai, Tween-80, Hydroxy-propyl methyl cellulose, Ploy ethylene glycol were purchased from Hi-media chemicals. All other chemicals and solvents used were of analytical grade.

METHODS: Soyabeanphosphatidyl choline with two different concentration of phospholipid content 70% & 30%, ethanol with 30 and 50% were selected for the initial screening studies. Based on the results of screening studies and with necessary modifications drug-loaded ethosome formulations were prepared using soya lecithin (70%) at three different levels 1, 2 and 3%, ethanol at 3 levels 1.5, 2.5 and 3.5% keeping drug concentration constant in all formulations. Ethosomes were prepared using modified mechanical dispersion method by dissolving drug and phospholipid in absolute alcohol under vigorous stirring at room temperature, water was added portion wise to pervious organic phase under constant stirring at 700 rpm. After complete addition of water, mixing was continued for 30 min at ambient temperature to obtain required ethosomal suspension. Suspension was allowed to cool at room temperature, sonicated for 3min in 2 successive cycles with 5 min rest between cycles and stored at room temperature for evaluation studies.

Optimization of Tacrolimus Loaded Ethosomes by 3^2 Central Composite designs: Ethosome dispersions were formulated according to a 3^2 central composite design, allowing the simultaneous evaluation of two independent variables -concentration of phospholipid and ethanol along with their interactions. The three different levels of these independent variables selected for phospholipid (X_1) were1, 2, 3% and 1.5, 2.5, 3.5% for Ethanol (X_2).The selected responses were particle size (Y_1), Deformability index (Y_2) and Entrapment Efficiency (Y_3).

Statistical Analysis: Polynomial equations developed for dependent variables particle size (Y1), DI (Y2) and EE% (Y3) were analyzed using Design Expert10.0. software which bear the form of equation-1:

$$Y = b_0+b_1X_1+b_2X_2+b_{12}X_1X_2+b_{11}X_{12}+b_{22}X_{22} \quad \ldots\ldots\ldots\ldots\ldots (1)$$

Where Y is dependent variable, b_0 arithmetic mean response and b_1 and b_2 are estimated coefficient for factor X_1 and X_2 respectively. Likewise b_{12}, b_{11} and b_{22} are the estimated coefficients for the interactions between X_1 and X_2, X_1 and X_1, X_2 and X_2 respectively. The main effects (X_1 and X_2) represent average result of changing one factor at a time from its low to high value. The interaction term (X_1X_2) shows how the response changes when two factors are simultaneously changed. The optimized composition and characterization of the ethosomal formulations are provided in **Table 1.**

TABLE 1: COMPOSITION AND CHARACTERIZATION OF ETHOSOME DISPERSIONS

Formulation Code	Conc of Phospholipid (%)		Conc of Ethanol (%)		Particle Size (nm)*	Deformability Index(DI)*	Entrapment Efficiency(EE)* (%)
	Actual values	Coded values	Actual values	Coded values			
ED1	1	-1	1.5	-1	278.9±0.004	4.80±0.13	67.33±1.12
ED2	1	-1	2.5	0	263.8±0.006	4.98±0.15	70.17±1.15
ED3	1	-1	3.5	+1	254.3±0.001	5.35±0.12	62.64±1.16
ED4	2	0	1.5	-1	221.8±0.005	5.66±0.11	76.35±1.12
ED5	2	0	2.5	0	213.2±0.003	5.96±0.14	82.57±1.16
ED6	2	0	3.5	1	209.8±0.007	6.39±0.15	73.11±1.15
ED7	3	+1	1.5	-1	271.6±0.006	6.72±0.13	86.37±1.14
ED8	3	+1	2.5	0	269.4±0.005	6.92±0.11	89.18±1.12
ED9	3	+1	3.5	+1	257.1±0.003	7.09±0.12	77.14±1.14

*****All values are reported as mean ± SD, n=3 measurements.

Ethosomes Characterization:

Encapsulation Efficiency Determination: Ethosomal dispersion was centrifuged at 15,000 rpm for 25 min and supernatant liquid was collected. 2 ml of supernatant liquid was transferred to 10ml volumetric flask, 0.5 ml of sulphuric acid was added and acetonitrile used to make up the volume. The solution was filtered and measured the absorbance using UV spectro-photometer at 291 nm. The % drug encapsulation efficiency was calculated by following formula.

EE% = Amount of entrapped drug / Total drug amount x 100

Vesicle size and Zeta Potential: The mean particle size (nm) and polydispersity index of the prepared ethosomes were measured by Malvern particle size zetasizer. The corresponding zeta potentials (mV) were determined by photon correlation spectroscopy using the same Zetasizer Nano instrument.

Deformability Index: Extrusion method was used to measure the elasticity of ethosomal vesicles. The Vesicle suspension were extruded through filter membrane (pore Index diameter 100 nm i.e. rp), by applying a vacuum of 2.5 bar [23].The quantity of vesicles suspension extruded in 5 minutes was measured (J). Vesicle size (rv) is measured after extrusion using Malvern Particle sizer. The deformability was reported as deformability index calculated by following equation:

$$\text{Deformability index} = j \times (rv/rp)^2$$

Where, j is the amount of suspension extruded in 5 min, rv the size of the vesicle after extrusion; rp the pore size of the membrane

Preparation of Fast Dissolving Ethosomal Films: Subglossal films (SGF) were prepared by solvent casting technique using HPMC K15M as film-forming polymer and Polyethylene Glycol 400 as plasticizer. Polymeric solution is prepared by dissolving specified amount of film forming polymer in 10 mL of the casting solvent (warm distilled water) with the necessary volume of the plasticizer. Ethosomal films were prepared by gently mixing the specified volume of the optimized ethosome dispersions (corresponding to the required tacrolimus dose) with the polymeric solution the specified amount of casting solution was transferred into a previously cleaned and dried glass moulds (area =18 cm^2, each 2 x2 cm^2 contains ethosomal dispersions consisting of 2.5 mg of drug). The formulated films were kept for 4 hrs at room temperature for the complete removal of solvent and dried in dessicator for 24 hrs and finally films were stored in dry place at ambient temperature by enwrapping in an aluminum foil to maintain the integrity and elasticity of the films till further evaluation. Formulated sublingual films were assessed for flexibility properties by determining the folding endurance value to come out for a best SL film [24-27].

Optimization of Subglossal Fast-Dissolving Ethosomal Films by Central Composite Design: HPMC K15 M (2–4% w/v) (X_1), PEG- 400 (0.1–0.3% w/v) (X_2) were selected as independent variables each at three different levels low (−1), medium (0), and high (+1) as they found to have impact on film formation and other film parameters. Nine runs of the experiment were evaluated for responses: disintegration time (Y1) and folding endurance (Y2). All other parameters like concentration of drug, method of preparation were kept constant to minimize fluctuations. The polynomial equation generated using Central Composite experimental design on Design-Expert® Software Version 10 is as follows:

$$Y=b_0+b_1X_1+b_2X_2+b_{12}X_1X^2_1+b_{11}X^2_1+b_{22}X^2 \ldots\ldots (2)$$

Where Y is dependent variable, b_0 arithmetic mean response and b_1 and b_2 are estimated coefficient for factor X_1 and X_2 respectively. Likewise b_{12}, b_{11} and b_{22} are the estimated coefficients for the interactions between X_1 and X_2, X_1 and X_1, X_2 and X_2, respectively. The main effects (X_1 and X_2) represent average result of changing one factor at a time from its low to high value.

TABLE 2: COMPOSITION AND CHARACTERIZATION OF OPTIMIZED FILMS

Formulation code	Conc of HPMC K15M (%)		Conc of PEG-400 (%)		Disintegration (sec)*	Folding endurance (no of folds)*
	Actual values	Coded values	Actual values	Coded values		
EF1	2	-1	0.1	-1	59±0.2	85±1.0
EF2	2	-1	0.2	0	53±0.1	90±1.0
EF3	2	-1	0.3	1	49±0.2	99±2.0
EF4	3	0	0.1	-1	43±0.4	129±2.0
EF5	3	0	0.2	0	40±0.1	157±1.0
EF6	3	0	0.3	1	39±0.1	178±1.0
EF7	4	1	0.1	-1	35±0.4	189±2.0
EF8	4	1	0.2	0	33±0.1	200±1.0
EF9	4	1	0.3	1	32±0.1	234±1.0

*All values are reported as mean ± SD, n=3 measurements.

The interaction term (X_1X_2) shows how the response changes when two factors are simultaneously changed.Predicted values were calculated based on equations derived and compared with experimental values to confirm the reliability and adequacy of the derived polynomial equations in predicting the responses. Composition and Characterization of optimized TAC ethosomal loaded subglossal films are provided in **Table 2.**

Evaluation of Fast Dissolving Films:

Physical and Mechanical Properties: Weight variation of the prepared films were studied using electronic Analytical balance (Acculab) by taking individual weights of ten randomly selected 4 cm^2 patches for each formulation prepared in different batches. Thickness was measured by micrometer screw gauge at different points of each formulation, and the mean values were calculated. The physical appearance was checked with visual examination of films and texture by touch. The films were evaluated for the other parameters like folding endurance, surface pH and drug content uniformity.

Folding endurance was determined by repetitive folding of the film at the same place till the film break, the number of times the film is folded without breaking was figured as the folding endurance value. *In-vitro* disintegration time was determined in a Petri dish containing 25 mL of phosphate buffer (pH 6.8) with whirling every 10 seconds. The disintegration time is the time recorded when the film starts to break or disintegrate [28-30].

Three films of each formulation were taken and kept in contact with 1 mL of distilled water for 1 hour at room temperature and the Surface pH was measured by bringing the electrode of the pH meter (microprogradmate), in contact with the surface of the film by equilibrating for 1 minute.

Drug content uniformity of the films was checked using 4 cm^2 patches, cut from different places in film and dissolving each film in 100 mL phosphate buffer (pH 6.8) [31]. The resulting solution was filtered, diluted with phosphate buffer if necessary and the absorbance was measured spectrophotometrically at 291 nm. The same procedure was repeated for at least three patches of each formulation, mean values and standard deviations were calculated. Characterization of different parameters of optimized ethosome-loaded sublingual films is provided in **Table 3.**

TABLE 3: PHYSICAL AND MECHANICAL PROPERTIES OF TAC- LOADED ETHOSOMES ENRICHED SUBGLOSSAL FILMS

Formulation code	*Thickness(mm)	*Weight (mg)	*Surface pH	*Drug Content
EF1	0.055± 0.3	42.19±1.23	6.9±0.12	94.68±1.21
EF2	0.059±0.3	40.23±1.54	6.3±0.13	93.35±2.53
EF3	0.062±0.4	42.17±1.16	6.9±0.13	92.24±1.33
EF4	0.057±0.2	41.42±1.37	6.8±0.11	94.25±1.92
EF5	0.056±0.2	43.18±1.23	6.9±0.13	95.68±1.44
EF6	0.053±0.2	42.48±1.78	6.4±0.19	93.19±1.76
EF7	0.060±0.3	41.68±1.32	6.9±0.12	97.57±1.71
EF8	0.067±0.1	40.29±1.45	7.0±0.15	97.29±1.54
EF9	0.069±0.1	49.14±1.42	6.8±0.13	98.57±1.35

*All values are reported as mean ± SD, n=3 measurements.

Attenuated Total Reflectance-Fourier transform Infrared Spectroscopy: The Fourier transform infrared spectroscopy (FTIR) spectra of the selected ethosomal film compared to its corresponding physical mixture and the individual solid components were recorded using ATR (Attenuated Total Reflectance) Spectroscopy.

Differential Scanning Calorimetry: Differential scanning calorimetry (DSC) scans were recorded for the selected ethosomal film, its corresponding physical mixture and the individual solid components. The samples were hermetically sealed in aluminum pans and heated at a constant rate of 10°C/min, over a temperature range of 25°C–200°C.

***In-vitro* Drug Release:** Drug release from ethosomal dispersion or from the prepared sublglossal ethosomal films (weighed and cut in area 4 cm^2) were performed using Franz diffusion cells with dialysis membrane as the artificial membrane using phosphate buffer at pH 7.4 in the donor compartment and pH 6.8 in the receptor

compartment on the magnetic stirrer with temperature set at 37°C and at speed of 100 rpm.Samples were withdrawn (5 mL) at the determined time intervals, centrifuged; supernatant was filtered and assayed using a UV-visible spectrophotometer (Shimadzu) at 291 nm. Samples were replaced by equal volumes of fresh buffer to maintain the same volume in the flasks [32]. The experiment was conducted in triplicates. The amount of drug released at each time interval was calculated, and the cumulative amount of drug released was calculated as a function of time to construct the drug release profile graphs. The release data were kinetically analyzed by curve fitting method to different kinetic models of zero-order, first-order, Hixoncrowell, Higuchi and Korsemeyer-Peppas models.

Stability Studies: Optimized films were checked for their stability by utilizing two storage conditions, one was normal room conditions and the other was 40°C/75% relative humidity. Fast dissolving film (E7) and optimized ethosomal subglossal film (EF9) were packed in butter paper followed by aluminum foil and plastic tape. After 8 weeks, fast dissolving film (E7) and optimized film (EF9) were evaluated for the physical appearance, surface pH and *in-vitro* drug release.

Scanning Electron Microscopy Studies: Selected fast dissolving ethosomal film (EF9) was subjected to SEM studies to observe for the surface characteristics using scanning electron microscope operated at an acceleration voltage from 200V to 30 kV.

***Ex-vivo* Permeability Studies:** In the present investigation, Franz diffusion cell was used to estimate the amount of drug permeated through the sublingual mucosa of goat for various samples namely pure drug- tacrolimus, plain film with drug, optimized TAC-loaded ethosomal sublingual film. A freshly excised sublingual mucosa of goat obtained from slaughter house was washed with distilled water, unwanted layers were removed and washed with phosphate buffer pH 6.8 without disturbing its integrity and stored properly for carrying diffusion studies. Tissue thus cleaned was clamped between the receptor and the donor compartment of Franz diffusion cell. The receptor chamber containing the sampling port, was filled with phosphate buffer pH 7.4, while donor chamber was filled with pH 6.8 phosphate buffer, by maintaining whole set up at 37 ± 0.5 °C with continuous stirring at a very low speed (50 rpm), using thermostatically controlled magnetic stirrer. Aliquot (2 ml each time) was withdrawn periodically at preset time from the receptor compartment which was filtered, diluted and evaluated spectrophotometrically to estimate the drug content. The volume of withdrawn sample was replaced with the buffer into the diffusion cell to keep the volume constant so that sink condition could be maintained. Experiment carried out up to 24 h with the excised sublingual tissue [33, 34].

Data Analysis: Data obtained from the permeability study for each formulation were used to calculate cumulative drug permeation (CDP), %CDP (mean ± standard deviation), permeability coefficient or apparent permeability (Papp) and Flux (J). Papp and *J* were calculated by using the standard formulae given in equations (3) and (4).

Permeability coefficient (apparent permeability);

$$Papp = (V_A/Area \times time) \times ([drug]_{acceptor}/[drug]_{donor})$$

Where, V_A = volume in the acceptor compartment, Area = surface area of the intestinal membrane, Time = total transport time

Flux;

$$J = Papp \times CD; \text{ ------- (4)}$$

Where, CD = Concentration of donor solution.

***In-vivo* Pharmacokinetic Studies using Wistar Rats:** The protocol of this research was reviewed and approved by the Institutional Animal Ethics Committee (IAEC) as per the CPSCEA guidelines (Approval no: AACP/IA/IAEC/MAR2019/06).All rats were maintained with standard diet. Rats were supplied with food and water *ad libitum.* Prior to the experiment, all animals were fasted for 18 hrs with free access to the water.

Animal Dosing and Sampling Scheme: The plasma concentrations of TAC were evaluated from healthy Wistar rats after the sublingual administration of the optimized ethosomal sublingual films (EF9) compared to the commercial Pengraf oral capsules (10 mg TAC).

Fifteen rats were used in the present study. The rats were fasted for 24 hours before the administration of the dosage forms. Approximately 2 mg/kg of the drug, corresponding to a 9-mg human dose, was used [35]. This equivalent dose for rats was calculated by the aid of surface area ratio, by using the following equation [36, 37].

AED (mg/kg) = Human dose (mg/kg) x Km ratio

Where, AED is animal equivalent dose and Km ratio is correction factor which is estimated by using the average body weight (kg) of animal species to its body surface area (m^2). Rats were randomly divided into three groups each of four animals as follows: the first group received normal saline by oral route, the second group was given the marketed dosage form (pengraf capsules) and the third group was given selected TAC sublingual ethosomal film (EF9). Multiple blood samples (1–2 mL) were collected in heparinized vacutainer tubes before administration and at 0, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12 and 24 hours following drug administration. The plasma was then separated after centrifugation and stored frozen at -20°C until further analysis [38].

Analysis of Plasma Samples: The collected samples of blood from the wistarrats were assayed with a modified HPLC method. In brief, 150 µL of blood samples from different groups of rats were mixed with a 250 µL of acetonitrile (precipitation reagent). The samples were allowed to stand for 30 min at 4ºC and then centrifuged at 14000 rpm for 15 minutes. 180 µL supernatant was fractionated by HPLC (model-Shimadzu), equipped with an ultraviolet (UV) detector.

Separation was achieved using zobax eclipse plus C-18 column (100 × 4.6 mm, internal diameter 3.5 µm). The elution was carried out at a flow rate of 1 mL/min using methanol: water (80: 20 v/v) as the mobile phase for the detection of tacrolimus in treated blood samples [39]. The Pharmacokinetic parameters Cmax, Tmax, AUC, Elimination rate constant and Absolute bioavailability were evaluated.

Histopathology Studies of Rat Sublingual Mucosa: Tacrolimus-loaded ethosomal fast dissolving films were administered sublingually into group of rats (n = 4). Rats were observed for mortality up to 48 h. After 48 h the rats were sacrificed and the sublingual mucosae excised inflated with a formaldehyde solution 4% (v/w) in physiological saline, fixed and stored at 4 °C until further histological examination [40].

RESULTS AND DISCUSSION:

Particle size, Deformability Index and EE % of the Prepared Ethosomes: TAC-loaded ethosomes were prepared by mechanical dispersion technique. Central composite design was utilized to optimize the ethosomal formulation and to arrive for the best formulations. Evaluation of the prepared ethosomes was carried out by measuring the particle size, Deformability index (DI) and EE.

Table 1 shows the particle size, DI and EE analysis of the freshly prepared and optimized ethosomes. The size of ethosomal formulations were in the permissible range 209.8-278.9nm reflecting the stability of the formed ethosomal dispersion. The Deformability index values for optimized ethosomes ranged from 4.80 to 7.09, as the ethanol concentration increased from 1.5 to 3.5% DI values increased which could be due to reduction of interfacial tension of the vesicle membrane providing elasticity to the vesicle membrane helping in penetration of active molecule through pores much smaller than their diameter [41].

The effect of different mass ratios of soya lecithin to ethanol on the EE of the drug in ethosomes is also shown in **Table 1**. Significant ($P<0.05$) highest EE 89.1 ± 0.1% was observed from formulation ED8 prepared at soyalecithin to ethanol mass ratio of 3:2.5. It has been observed that as phospholipid concentration increased encapsulation efficiency (EE) found to be increased but EE found to be decreased with increase in ethanol concentration which might be attributed to higher permeation enhancing property of ethanol leading to the leakage of lipid bilayer and high concentration of ethanol cannot co-exist with lipid vesicles which might have significantly affected the EE of Ethosomes .Suitable particle size, DI and EE were obtained from ethosomal dispersion formulation EF8 with 3:2.5 phospholipid to ethanol ratio and thus it was selected for the preparation of the ethosomal film.

Polynomial Equations Obtained by 3^2 Central Composite Design for Tacrolimus Loaded Ethsosme Dispersion:

Particle Size = $214.33 + 0.33X_1 - 8.33X_2 + 2.50X_1X_2 + 51X_1^2 + 0.000X_2^2$…….. (5)

DI = $6.02 + 0.94\ X_1 + 0.25\ X_2 - 0.082\ X_1X_2 - 0.025\ X_1^2 - 0.030\ X_2^2$ ……..… (6)

EE=$81.93+8.83\ X_1+2.93\ X_2-1.04\ X_1X_2-1.94\ X_1^2-6.88\ X_2^{2...}$…… (7)

As per the response surface curves and polynomial equations generated by CCD optimization design phospholipid concentration found to have positive effect on vesicle size *i.e* as concentration of phospholipid increased vesicle size found to be increased and ethanol had negative effect on particle size which were in accordance with experimental studies carried out.

Concentration of soyalecithin and ethanol had profound positive influence on Deformability and EE of formulated vesicles suggesting the significant effect of independent variables on dependent factors as seen in **Fig. 1, 2** and **3.**

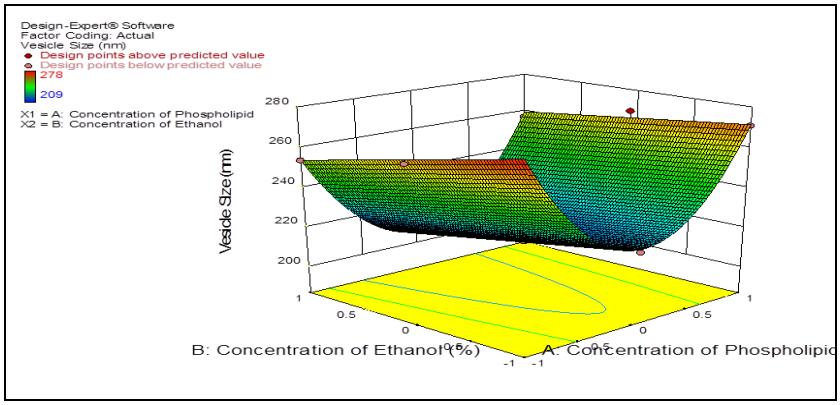

FIG. 1: EFFECT OF INDEPENDENT VARIABLES ON THE VESICLE SIZE

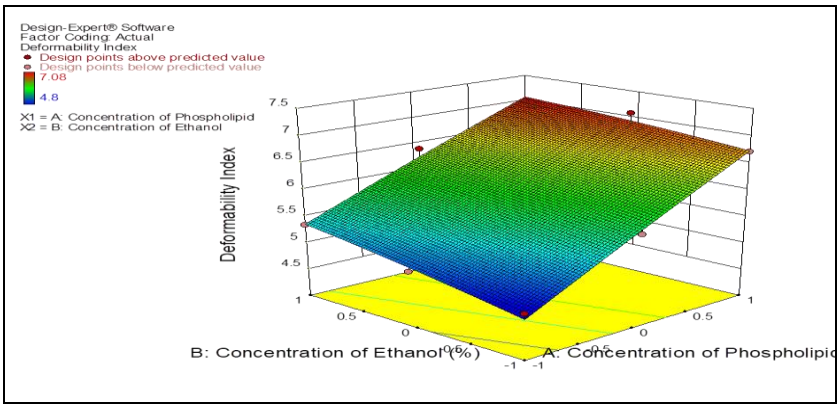

FIG. 2: EFFECT OF CONCENTRATION OF ETHANOL AND SOYALECITHIN ON DEFORMABILITY INDEX

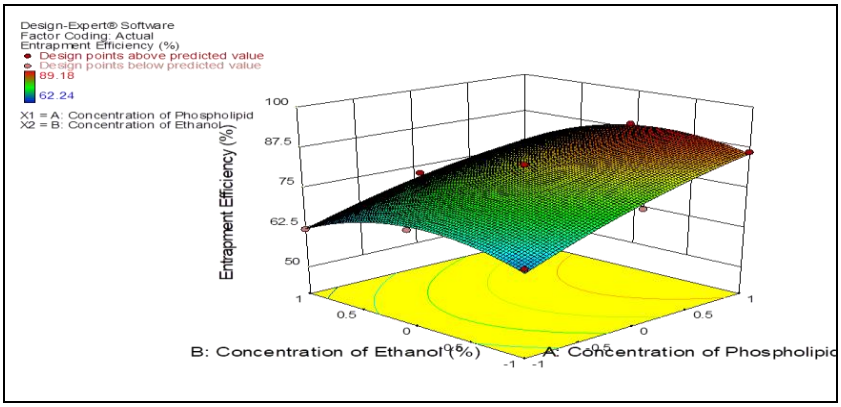

FIG. 3: EFFECT OF ETHANOL AND PHOSPHOLIPID ON THE ENTRAPMENT EFFICIENCY

Evaluation of fast Dissolving Films:

Physical and Mechanical Properties: The prepared ethosomal films were found to be homogenous, transparent and flexible with smooth finish. The results of other physical characteristics such as weight, thickness uniformity, film flexibility, surface pH, uniformity of drug content and *in-vitro* disintegration time are presented in **Table 2** and **3**. All films had mean weight ranged from 40.23±1.54 to 49.14±1.42mg and mean thickness in the range of 0.055± 0.3 to 0.069±0.1mm with nonstatistically significant difference (P>0.05) in both weight and thickness among the various formulations.

The mean values of surface pH of all prepared films were close to neutral (6.3±0.13 to 7.0±0.15) with nonsignificant differences obtained (P>0.05). At this pH, the films found to be less irritant to the mucosal lining of the oral cavity making application of the film easy and comfortable. Nonsignificant difference (P>0.05) was observed in the drug content of TAC among the prepared ethosomal films (92.24±1.33to 98.57±1.35). All the formulations were found to contain almost uniform quantity of drug indicating reproducibility of the technique. All the prepared films were highly flexible with folding endurance values >200 times without cracking.

The mean *in-vitro* disintegration time for the prepared films containing HPMC K15M film forming polymer was found to be in the range of 32±0.1 seconds to 59±0.2 seconds indicating the fast dissolving nature of prepared ethosomal sublingual films.

Polynomial Equations of Quadratic Model for Optimized Ethosomal fast Dissolving Sublingual Film (EF9):

Disintegration time = $40.11-10.17X_1-2.83X_2+1.75X_1X_2+2.83X_1^2+0.83\ X_2^2$…….(8)

Folding endurance= $152.44+58.17\ X_1+18.00\ X_2-7.75\ X_1X_2-5.17\ X_1^2+3.33\ X_2^2$ ……..(9)

3D graphs and polynomial equations generated by CCD optimization depicted that film forming polymer as well as plasticizer had profound negative effect on DT time of ethosomal fast dissolving sublingual film. As the Concentration of HPMCK15M and PEG-400 increased DT time was found to be decreased which is very well seen in the formulations prepared **Fig. 4**. But in the case of folding endurance both film forming polymer and plasticizer found to have positive effect **Fig. 5** indicating optimum concentration of film forming polymer and plasticizer is necessary in order to obtain robust yet smooth films.

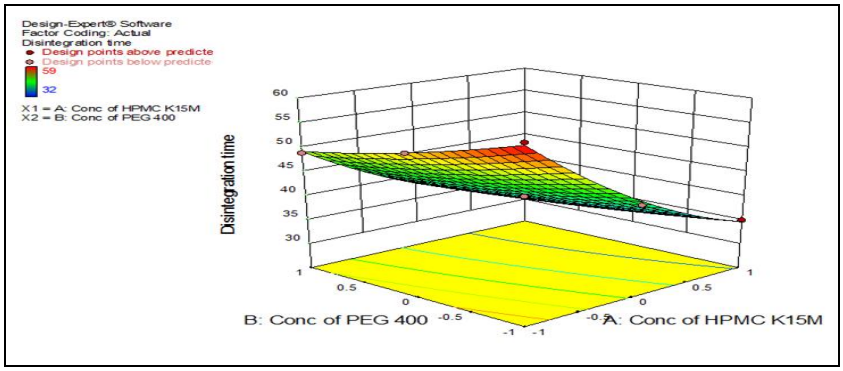

FIG. 4: EFFECT OF FILM FORMING POLYMER AND PLASTICIZER ON DT OF SUBGLOSSAL FILMS

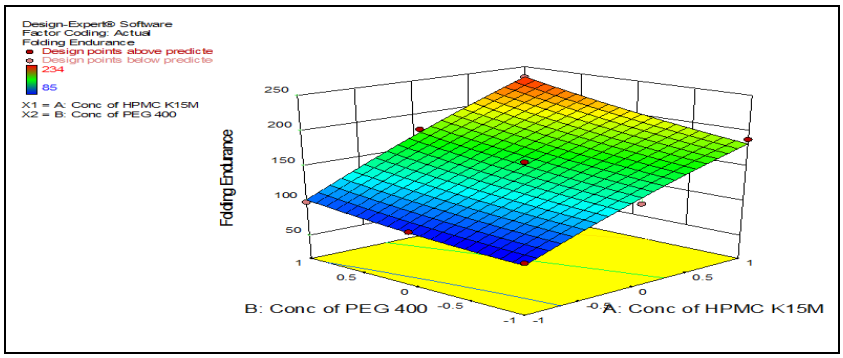

FIG. 5: RESPONSE SURFACE GRAPH DEPICTING THE EFFECT OF HPMC K15M AND PEG 400 ON

Folding Endurance of Films: Zeta potential reveals the surface charge of colloidal dispersions as it usually predicts the dispersion stability. Zeta potential of the final optimized formulation of tacrolimus was conducted to prove the stability of prepared nanovesicles. Negatively charged particles contribute to the high stability of the colloidal solution as columbic repulsive forces between the particles prevent them from agglomerating in the colloidal state thereby stabilizing the particle suspensions/ emulsions. As shown in **Fig. 6** optimized ethosomal vesicle exhibited negative zeta potential values as -31.4 mV. The observed negative potential may be attributed to the negative charge on the polar head group of the phospholipid, thus helping in prevention of aggregation of vesicles and maintaining a homogenous suspension.

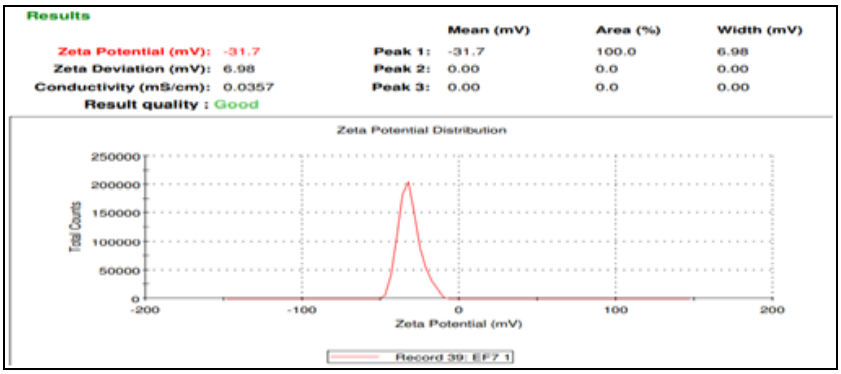

FIG. 6: ZETA POTENTIAL OF OPTIMIZED TAC-LOADED ETHOSOMAL SUBLINGUAL FILM (EF9)

Differential Scanning Calorimetry: DSC provides information on melting, crystallization, decomposition or a change in heat capacity. It is useful in assessing the physicochemical status of the entrapped drug and the interaction among different components. **Fig. 7, 8** and **9** illustrates the DSC thermograms of the pure drug, physical mixture and selected TAC ethosomal film (EF9). The DSC scan of free tacrolimus showed single sharp endothermic melting peak, at 131.98°C which corresponds to the melting transition of the drug. Data indicate the crystalline nature of the drug and are in good agreement with previously reported melting transitions of thermal analysis of TAC [44].

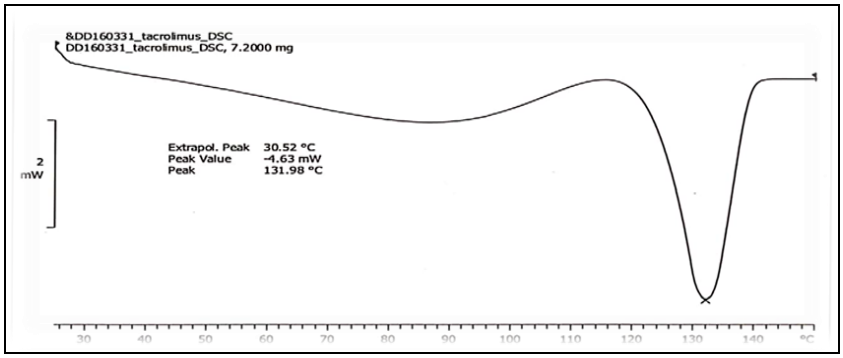

FIG. 7: DSC OF PURE DRUG

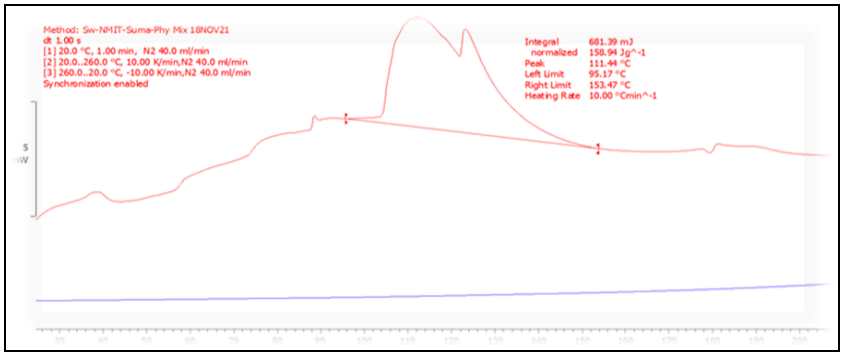

FIG. 8: DSC OF PHYSICAL MIXTURE

Concerning the corresponding physical mixture of tacrolimus and soyalecithin, phospholipid showed sharp peak at 175.2°C with reduced intensity of drug peak. However, this endothermic peak was not found in thermogram of the ethosomal sublingual film confirming the entrapment of the drug in vesicular system within the formed film.

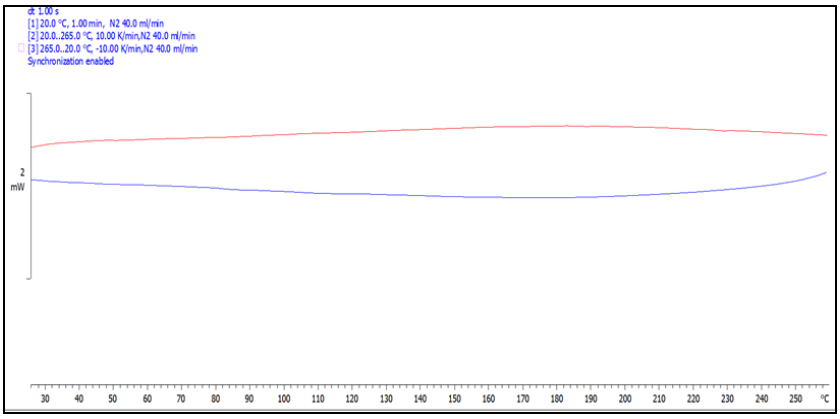

FIG. 9: DSC OF OPTIMIZED SUBLINGUAL FILM (EF9)

Fourier Transform Infrared Spectroscopy: Fig. 10, 11 and **12** displays the FTIR spectra of the pure drug, corresponding physical mixture and optimized TAC ethosomal film (EF9).The spectrum of the pure drug showed characteristic peaks at 1020, 10352 (C-O stretching), 3350, 3451 (NH stretching), 1,451 (C=C group), 2903, 2977 and 3281 (OH stretching). All these characteristic peaks of the pure drug were also found in the FTIR spectrum of ethosomal film and in the spectra of the physical mixtures. Attenuated Transmittance Reflectance analysis revealed that there was no predominant chemical interaction of TAC with ingredients of the selected prepared ethosomal film as indicated by the presence of the essential bands and the absence of bands for new functional groups.

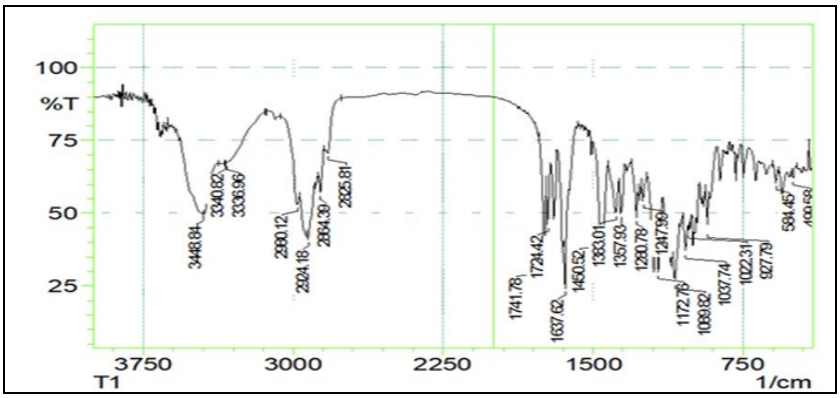

FIG. 10: FT IR OF THE PURE DRUG

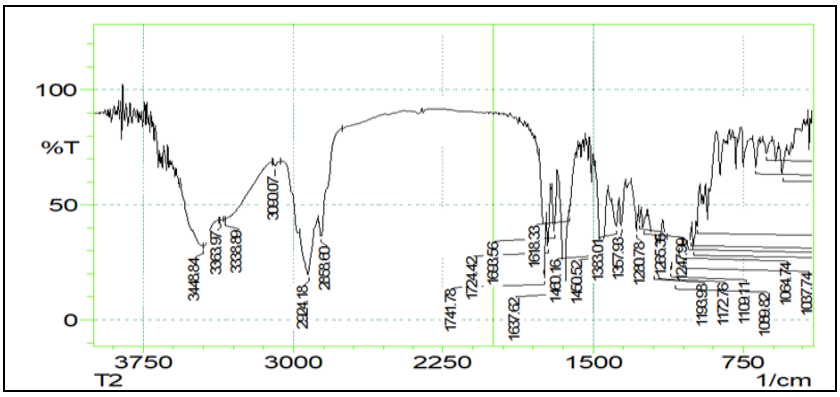

FIG. 11: FT IR OF THE PHYSICAL MIXTURE

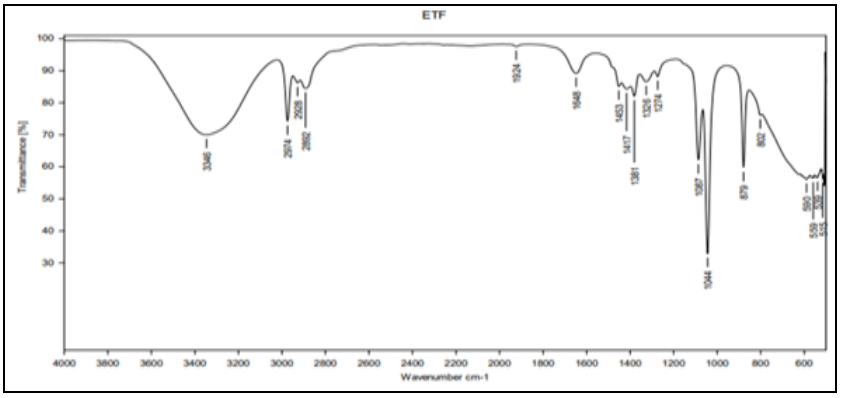

FIG. 12: FT IR OPTIMIZED TACROLIMUS LOADED ETHOSOMAL SUBLINGUAL FILM (EF9) BY ATR

***In-vitro* Drug Release:** Sublingual administration of TAC fast dissolving film (EF9) is expected to enhance drug bioavailability through the avoidance of first-pass hepatic metabolism. In addition, inclusion of drug into ethosomes prior to formation of the film, allows delivery of therapeutically significant levels of the drug over prolonged time. **Fig. 13** illustrates the cumulative TAC release from the selected ethosomal fast dissolving film (EF9) in comparison with its ethosomal dispersion (ED8). Incorporation of the medicated ethosomes within the fast dissolving film base did not significantly alter (P>0.05) the drug release from the dispersed ethosomes. But the usage of sublingual films can be the convenient dosage form for the administration to patients with short residence time in the mouth avoiding absorption of the drug loaded ethosomes through the mucosa.

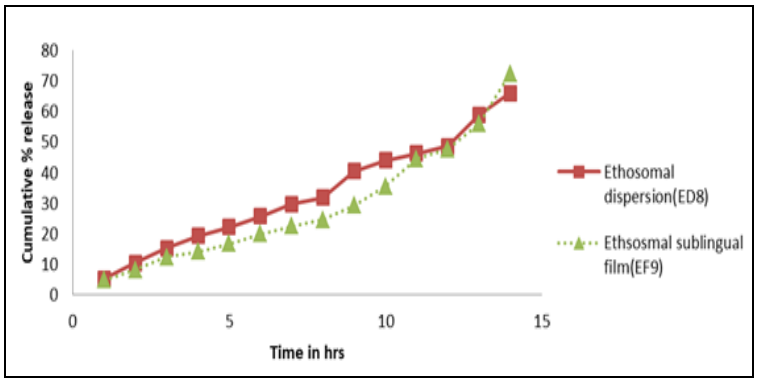

FIG. 13: CUMULATIVE TAC RELEASE FROM ETHOSOMAL FAST DISSOLVING FILM (EF9) IN COMPARISON WITH ITS ETHOSOMAL DISPERSION (ED8)

Kinetics of Drug Rrelease: Release kinetic parameters and correlation coefficients (R^2) calculated for optimized films are summarized in **Table 4**. The *in-vitro* release data indicate that the release of drug from optimized sublingual ethosomal film follows zero order with diffusion-controlled mechanism (Higuchi model) according to the higher correlation coefficient. To have meticulous understandings regarding release of drug from formulated nanovesicle loaded subglossal film korsemeyer–Peppas model was applied. The diffusion exponent of Korsemeyer–Peppas model Peppas model was found to be less than 0.5, indicating the Fickian transport and drug release being controlled by slower degradation of nanovesicular bilayers [42].

TABLE 4: DRUG RELEASE KINETICS FROM OPTIMIZED SUBGLOSSAL FILM

Formulation	Correlation coefficient (R^2)*					
	Zero Order	First Order	Higuchi	Hixson Crowell	Peppas	KorsmeyerPeppas equation (n)*
Ethosomal dispersion (ED8)	0.9801±0.19	0.695±0.7	0.9352±0.01	0.8712±0.019	0.9012± 0.016	0.3145±0.012
Ethosomal film (EF9)	0.9905±0.012	0.6754±0.15	0.9783±0.01	0.8863±0.017	0.9008±0.013	0.381±0.014

* All values are reported as mean ± SD, n=3 measurements.

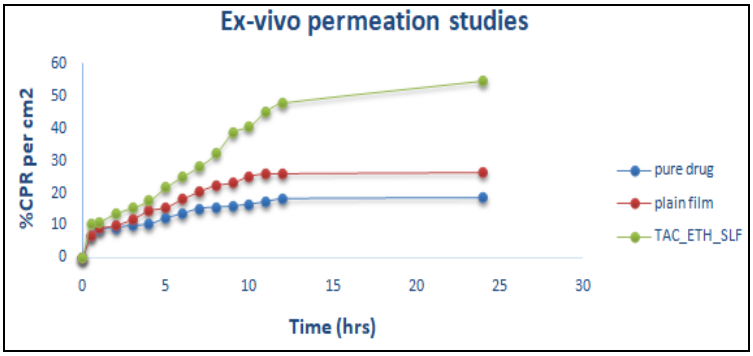

FIG. 14: COMPARATIVE *EX-VIVO* PERMEATION STUDIES BETWEEN PURE DUG, PLAIN FILM AND OPTIMIZED SUBGLOSSAL FILM

TABLE 5: STEADY STATE FLUX PARAMETERS OF THE PURE DRUG, MEDICATED FILM AND ETHOSOMAL SUBLINGUAL FILM

Parameters	Pure drug suspension (±SD)*	Plain film with drug (±SD)*	TAC-loaded Ethosomal sublingual film (±SD)*
Steady state Flux (µg cm^2/ /hr)	0.18±0.01	0.26±0.05	2.9±0.01
Flux	0.899±0.01	3.7±0.01	37.54±0.05
Permeability(cm/hr)	0.04±0.03	0.05±0.03	0.464±0.04
Apparent Permeability	1.94±0.04	3.36±0.06	6.38±0.03

* All values are reported as mean ± SD, n=3 measurements.

Stability Studies: Selected fast dissolving film (E7) and optimized drug-loaded subglossal film (EF9) were subjected for stability studies at normal temperature and 40°C for 30 days to assess their stability as summarized in **Table 6**. After 30 days, stored film formulations were checked for the changes in the physicochemical parameters (weight, surface Ph and folding endurance), nonsignificant differences ($P<0.05$) were observed both at normal temperature and elevated temperature (40°C) compared with initial readings at zero days.

TABLE 6: PHYSICOCHEMICAL EVALUATION OF THE SELECTED OPTIMIZED FAST DISSOLVING FILM FORMULATIONS DURING STABILITY STUDY

Parameters	Fast dissolving film(F7)			Optimized ethosomal film (EF9)		
Days (temperature)	Zero*	60(normal temperature)*	60(40^0c)*	Zero*	60(normal temperature)*	60(40^0c)*
Weight(mg)*	51.3±0.13	49.2±0.15	45.5±0.3	49.14±1.4	44.24±0.4	41.67±0.4
Surface pH*	7.4±0.16	7.8±0.7	7.2±0.6	6.8±0.13	6.5±0.5	6.4±0.8
Folding endurance (no of folds)*	198±0.3	196±1.34	189±1.67	234±1.0	210±1.46	197±1.45

* All values are reported as mean ± SD, n=3 measurements.

Scanning Electron Microscopy: For the selected and optimized ethosomal fast dissolving film, even textured globular vesicles were seen with high dispersibility without any perceptible mass confirming the intactness of formulated optimized subglossal film without any significant changes in terms of morphology, shape and dispersibility.

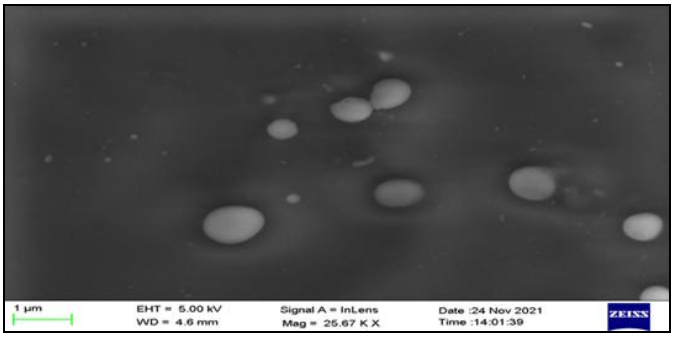

FIG. 15: SURFACE MORPHOLOGY OF SELECTED OPTIMIZED ETHOSOMAL SUBLINGUAL FILM EF9

Pharmacokinetics and Bioavailability of TAC After Sublingual and Oral Administration: Small particle size, short *in-vitro* disintegration time, high EE and favorable zeta potential contributed to the selection of optimized film (EF9) for further *in-vivo* studies.

Witsar rats were used to assess the plasma levels of sublingually absorbed TAC from ethosomal film (EF9) in comparison to the oral TAC capsules, pengraf. TAC plasma concentration was measured using a sensitive high-performance liquid chromatography assay. The mean plasma drug pharmacokinetic data based on the plasma concentrations are summarized in **Table 7**.

It could be noticed that the sublingual formulation of tacrolimus loaded ethosomal films exhibited controlled and sustained release with higher peak serum concentration (Cmax) of the drug in the sublingually treated animals 75.19±0.001 ng/mL at Tmax of 2±0.03hours than that of the oral one (33.86±0.001ng/mL). This reflects the higher rate of controlled absorption after sublingual delivery of the drug with significant increase in the area under the plasma concentration time curve (AUC) (1926±0.001h mL-1) as compared with the AUC obtained after administration of the oral capsule (570.4± 0.005h mL-1).

This could be due to the ingress of drug into the systemic circulation directly to exert its action at a controlled rate due to the slow erosion rate of the ethosomal bilayers with enhanced bioavailability compared with that oral dosage form.

The narrow AUC obtained after the oral administration of the drug reflects the rapid disappearance of the drug from the plasma due to its short half-life (12.4± 0.002hrs) compared with that after sublingual ethosomal film administration (23.9±0.002hrs).The comparative results is shown in **Fig. 16.**

TABLE 7: PHARMACOKINETIC PARAMETERS OF TAC IN PLASMA AFTER SUBLINGUAL ADMINISTRATION OF ETHOSOMAL FILM COMPARED TO COMMERCIAL ORAL CAPSULES

Parameters	Pengraf CAP	TAC ethosomal film(EF9)
Cmax (ng/ml±SD)*	35.7± 0.002	75.19±0.001**
Tmax (h±SD)*	0.25± 0.01	2±0.03**
Half-life (h±SD)*	12.4± 0.002	23.9±0.002**
Elimination rate constant (Kel)*	0.055± 0.003	0.028±0.001**
AUC0_inf(0-24hr(±SD)*	570.4± 0.005	1926±0.001**
Absolute Bioavailability*(F)%	21±0.001	91±0.003**

All values are reported as mean ± SD, n=3 measurements **Significant differences were achieved related to that of the oral capsules.

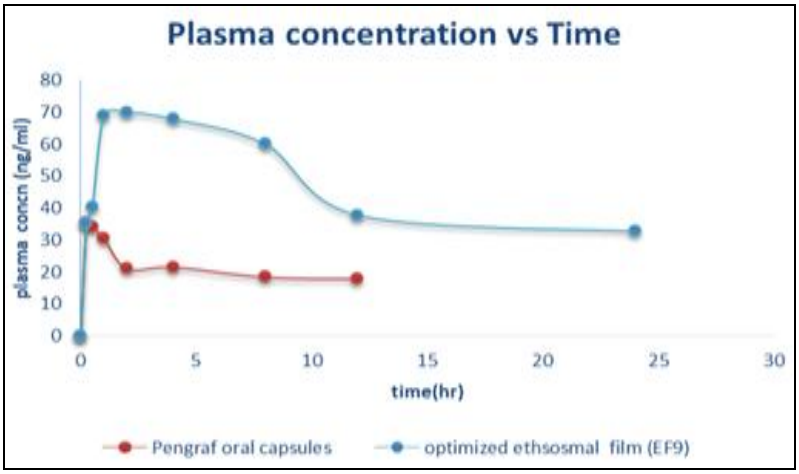

FIG. 16: PLASMA CONCENTRATIONS OF TAC AFTER SUBLINGUAL AND ORAL ADMINISTRATION

Histopathology Studies: Histopathology studies were conducted to check the toxicity of formulated vesicular sublingual film. As seen in the slides **Fig. 17, 18** the cells in all the slides found to be intact with no inflammation indicating the safety of the formulated vesicular sublingual film.

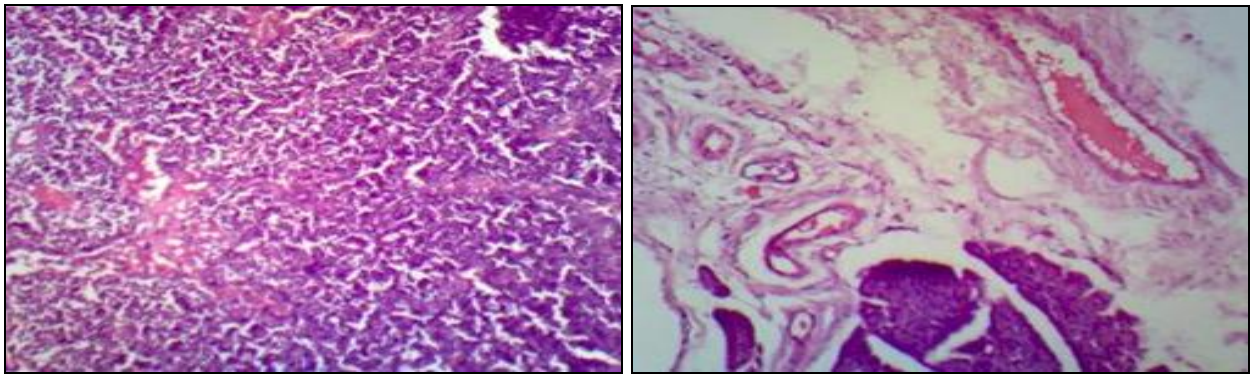

FIG. 17: HISTOPATHOLOGY STUDIES OF SUBLINGUAL TISSUES OF NORMAL CONTROL GROUP RAT (WITHOUT THE DRUG)

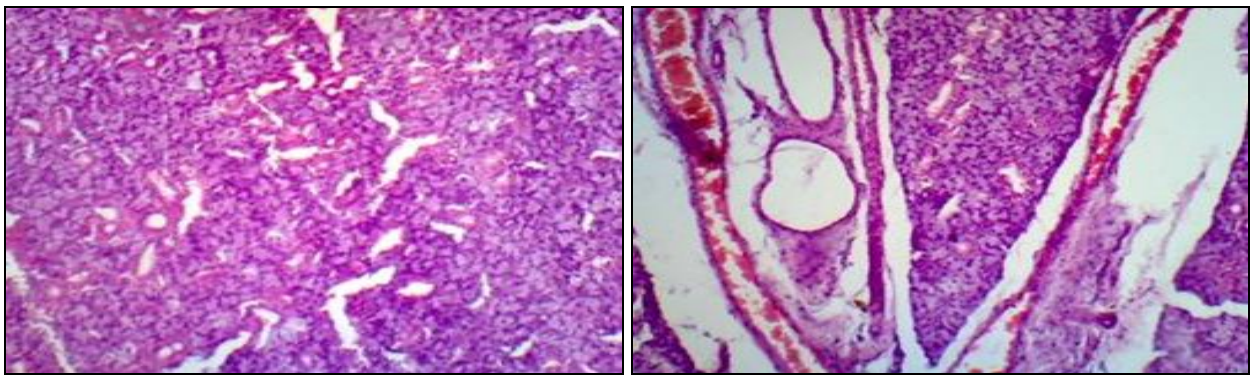

FIG. 18: HISTOPATHOLOGY STUDIES OF RAT SUBLINGUAL TISSUES OF OPTIMIZED TAC-LOADED ETHOSOMAL SUBLINGUAL FILM

CONCLUSION: Subglossal fast dissolving films containing TAC-loaded ethosomes were formulated, optimized and evaluated for their abilities to enhance bioavailability of tacrolimus. Central composite design was applied to achieve robust formulations with reproducible results. Drug-loaded ethosomes with small size, high deformability index and EE were selected for incorporation into optimized subglossal films, which were then evaluated for different physical characteristics.

Ex-vivo, *in-vitro* and *in-vivo* characterizations were carried out to check the drug release from nanovesicles. The optimal ethosomal film showed sustained release of the drug compared to the pure drug and medicated film containing the free drug. The *ex-vivo* release kinetics of drug from the nanovesicular film followed the Higuchi model indicating diffusion mechanism with bioerosion of lipid bilayers for controlled drug release. *In-vivo* study in rats showed significantly extended and controlled drug release from ethosomal film compared to that of marketed dosage form. Hence, it can be concluded that the prepared subglossal fast dissolving ethosomal film could be an efficient drug delivery system in sustaining the therapeutic concentrations of tacrolimus and thereby improving the patient compliance in organ transplanted patients.

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CONFLICT OF INTEREST: The authors declare no conflict of interest

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