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Formulation and *In Vitro* Evaluation of Topical Ethosomal Gel Containing 5-Fluorouracil

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ABSTRACT

The aim of present study was to develop and evaluate the topical delivery of a novel vesicular carrier, ethosomes having 5-fluorouracil, an anticancer drug followed by incorporation into carbopol gel. Ethosomes of 5-Fluorouracil were prepared by cold method using phospholipids, ethanol and propylene glycol. 5-Flurouracil loaded ethosomal vesicles were characterized for vesicular shape and surface morphology, vesicular size, entrapment efficiency, zeta potential and FTIR studies. Transmission electron microscope (TEM) imaging showed a spherical with a smooth surface and almost homogenous structure for ethosomes. The prepared ethosomal formulations of 5-fluorouracil with different phospholipids have shown average particle size in the range of 202.3 ± 3.4 to 202.3 ± 7.1 nm, polydispersity index in the range of 0.37 to 0.41, zeta potential -1.35 ± 0.35 to -26.9 ± 0.98 mV and % entrapment efficiency in the range of 87.9 ± 1.7 % to 88.7 ± 2.3 %. FTIR study concluded that no major interaction occurred between drug and phospholipids used in the present study. The ethosomal formulations were incorporated into the 1% carbopol 980 gel and evaluated for *in vitro* drug release. *In-vitro* drug release through the cellophane membrane showed the release of drug from ethosomal gels was enhanced as compared to plain drug solution.

Keywords: Topical delivery, gel, ethosomes, phospholipids, 5-fluorouracil

INTRODUCTION

Skin cancer is the one of the most common type of cancer with more than one million newly diagnosed cases in the United States, annually[1]. There are three forms of skin cancer as (a) Basal cell carcinoma (BCC); (b) Squamous cell carcinoma (SCC); (c) Melanoma affecting melanocytes. BCC and SCC are known as non-melanoma skin cancers. Non-melanoma skin cancers are not lethal but can destroy sensory organs such as nose, ear and lips[2]. In contrast, melanoma skin cancers are an aggressive type that can metastasize and cause death. Melanoma has a much higher death rate than non-melanoma skin cancers[3, 4]. There are some methods used for treatment of skin cancer like surgery, radiation and chemotherapy. Chemotherapy with drugs like cisplatin, 5-fluorouracil, doxorubicin and mitomycin are used for treatment of SCC and BCC. Paclitaxel either alone or in combination with cisplatin or carboplatin, a combination of cisplatin, vinblastin, dacarbazine or dacarbazine, carmustine, cisplatin and tamoxifen are used in treatment of melanoma. To increase the patient compliance and to reduce the surgical costs and undesirable scars particularly in cases where the cancer has spread over large areas of the body, topical administration of anticancer drug has been more preferred. Topical administration of any anticancer drug is an interesting alternative for reducing side effects, for increasing drug targeting and therapeutic benefits. But it has a drawback like poor penetration of drug in cancerous site. Thus, the major problem of this kind of treatment is to increase penetration of any anticancer drug in sufficient level to kill tumor cells. Several techniques and formulations like physical enhancers, chemical enhancers and use of suitable

carriers have been used to overcome skin barriers and to reach the skin malignancies by favoring drug penetration into deeper layer into skin. Vesicular drug delivery system as carriers could improve skin targeting, improving the drug's ability to reach and penetrate into tumor cells. Moreover, vesicular drug delivery system can improve drug stability and reduce skin irritation by avoiding direct of the drug with skin's surface. Liposomes are most widely used vesicular drug delivery system for treatment of skin cancer. Traditional liposomes are composed of phospholipid. It was evident that traditional liposomes are of little or no values as carriers for transdermal drug delivery because they do not deeply penetrate skin but remained confined to upper layer of the stratum corneum[5]. There was some change in the formulations of liposomes by addition of different components like ethanol, nonionic surfactant and other amphiphiles. These new generation of liposomes called elastic liposomes, ultra deformable and ultra flexible liposomes. Transferosomes, niosomes and ethosomes were named given to the first, second and third flexible liposome generations, respectively.

In recent years, ethosomes have become new liposome carriers with high deformability; high entrapment efficiency and good transdermal permeation rate in the drug delivery system and are suitable for transdermal administration[10, 11]. Ethosomes are soft malleable vesicles which are mainly comprises of phospholipids, short chain alcohol (mainly ethanol) at relatively high concentration and water. It was first put forward by Touitou E in 1996 and was designed for enhanced delivery of drug into skin[7, 8]. Ethosomes are shown to entrap drug molecules with various physicochemical characteristic i.e., of hydrophilic, lipophilic or amphiphilic more efficiently than liposomes[9]. Size of ethosome vesicles varied from tens of microns to nanometers.

High concentration of ethanol makes the ethosome unique, as the ethanol is known for, its disturbance of lipid bilayer organization, therefore when integrated into a vesicle membrane, it gives that vesicle the ability to penetrate the stratum corneum. Also, because of their high concentration, lipid membrane is packed less tightly than conventional vesicles but has equivalent stability allowing a more malleable structure and improved drug distribution ability in the stratum corneum lipids. Ethosomes were reported to improve in vitro and in vivo skin delivery of various drugs. Contrary to deformable liposome, ethosomes are able to improve skin delivery of drugs both under occlusive and non-occlusive conditions[12]. Ethanol acts as an efficient permeation enhancers that are believed to act by affecting the intercellular region of the stratum corneum[6].

5-Fluorouracil, an antineoplastic drug, is a pyrimidine analogue that showed a broad spectrum of activity against several solid tumors by interfering with thymidylate synthesis[13, 14]. It is an acidic, water-soluble hydrophilic drug which has 28-100% bioavailability and 10-20 min half life[16, 17]. It is one of the oldest antineoplastic drugs which are used for treatment of solid cancers like colorectal, stomach, liver, brain, breast and pancreas. It also used in some skin cancers such as AK and NMSC as a topical formulation. Per-oral 5-FU administration results in poor drug absorption, variable first pass elimination by gut and liver and erratic bioavailability. After intravenous administration, fluorouracil will produce the severe systemic toxic effects of gastrointestinal, hematological, neural, cardiac and dermatological origin[15]. To reduce the systemic toxic effects, topical route can be more preferred in the form of creams and gels.

The aim of the present study was to develop topical ethosomal gel of fluorouracil and to evaluate it with respect to various in vitro parameters. In this paper, ethosomes with phospholipids and ethanol were prepared and evaluated for particle size, morphology, zeta potential, entrapment efficiency and in vitro drug release study.

MATERIALS AND METHODS

Materials

5-Fluorouracil was provided as a gift sample from Cipla Limited, India. Leciva 70 and leciva 90 were purchased from Vav Life Sciences Pvt. Ltd., Mumbai, India. Ethanol obtained from Changshu Yangyuan Chemical, China. Carbopol 980 was obtained from Lubrizol Advanced Material, Europe. Propylene glycol and potassium dihydrogen phosphate were obtained from Thomas Baker (Chemicals) Pvt. Ltd., Mumbai, India. Sodium hydroxide was purchased from Central Drug House Pvt. Ltd., Mumbai, India. Triethylamine obtained from Universal Laboratories Private Ltd. All the materials used in this study were of analytical and pharmaceutical grade.

Method of preparation

Ethosomal formulation was prepared according to the method reported by the Touitou et al. In this method, phospholipid and fluorouracil was dissolved in ethanol in a covered vessel at room temperature by vigorous stirring (Table **1**). This mixture was heated to 30ºC in a water bath. The water was heated to a 30ºC in a separate vessel and was added to the mixture drop wise in the center of vessel which was stirred for 5 min. at 700 rpm in a covered vessel. The vesicle size of ethosomal formulation can be decreased to a desired extend using sonication method. Finally, the formulation is stored under refrigeration.

Table 1: Composition of Ethosomal formulations

S. No.	Formulation code	Drug(mg)	Lipid	Drug: lipid ratio
1.	EL70	100	Leciva 70	1:10
2.	EL90	100	Leciva 90	1:10

Characterization of Ethosomes

Vesicular shape and surface morphology

Vesicular shape of the ethosomal preparations were investigated by using a transmission electron microscope (TEM) with an accelerating voltage of 80KV. Ethosomal suspension diluted with water was mounted on a copper grid and the excess solution was removed with a filter paper. The grid was covered with a small drop of staining solution (1%w/v phosphotungstic acid). It was left on the grid for few

minutes and excess solution was drained off with filter paper. The grid was allowed to dry thoroughly in air and sample was examined in transmission electron microscope (Model, Hitachi 7500).

Vesicle size and size distribution

The vesicles size of each ethosomal suspension was measured by dynamic light scattering technique using computerized inspection system (Malvern Zetasizer, Nano-ZS, Malvern Instruments Ltd.) at 25 ± 1°C. For vesicle size measurement, vesicular suspensions were diluted with distilled water and the measurements were conducted in triplicate.

Polydispersity index

Polydispersity index is a parameter to define the particle size distribution of ethosomes. a measure of particle size distribution, the polydispersity index ranges from a value of 0.01 for monodispersed particles and up to values of 0.5-0.7. Samples with very broad size distribution have polydispersity index values > 0.7.

Determination of percentage entrapment efficiency

Ethosomes preparations containing Fluorouracil was estimated by ultracentrifugation technique for measuring its entrapment efficiency. The total volume of ethosomal suspension was measured and 1ml of this formulation was transferred to 10ml centrifuge tube. The suspension was diluted with phosphate buffer pH 6.8 up to 5ml. Ethosomes were separated by ultracentrifugation at 20000 rpm for 1 hr. (Remi Instruments Ltd., Mumbai). Supernatant and sediment were recovered and their volume was measured. Supernatant was analyzed for drug content after suitable dilution with phosphate buffer solution by measuring absorbance at 265 nm using Shimadzu UV Spectrophotometer. The method was repeated at least three times. Entrapment efficiency was calculated by using the formula:

$$\mathbf{EE} = \frac{\mathbf{Qt - Qs}}{\mathbf{Qt}} \times \mathbf{100}$$

Where EE is the entrapment efficiency, Qt is the theoretical amount of 5-FU that was added and Qs is the amount of 5-FU detected only in supernatant.

Zeta potential determination

Zeta potential of vesicles was determined using Zetasizer (Nano-ZS, Malvern Instruments) at 25±1°C. Prior to the measurements, vesicular suspensions were diluted with distilled water and injecting into zeta potential measurement cell. The measurements were made in triplicates.

Fourier Transform Infrared Spectroscopy (FTIR)

To investigate any possible interaction between drug and the vesicle membrane component (phospholipids), IR spectral studies of pure drug and its formulations EL70 and EL90 were carried out by using FTIR spectrophotometer (Perkin Elmer Spectrum 400) and spectrum was recorded in region of 4000 to 450 cm^{-1}. Interaction between drug and excipients was indicated either by presence of additional peaks or by absence of characteristic peaks in the IR spectrum of formulations that corresponds to the drug. The procedure consisting of dispersing a sample in KBr and compressing into disc by applying a pressure of 5 tons for 5 min. in a hydraulic press. The pellet was placed in the light path and spectrum was obtained. All spectra were collected as an average of three scans at a resolution of 2 cm^{-1}.

Preparation of topical gel containing ethosomes

Carbopol 980 (1% w/v) was added in minimum amount of distilled water and kept for an hour. Ethosomal suspension (15ml) containing 5-FU (100mg) was slowly added in the carbopol gel with continuous stirring at 700rpm in a closed vessel and maintained at temperature 30°C until homogeneous ethosomal gels (EG-L70 and EG-L90) were achieved. The pH was then adjusted to neutral using triethylamine and stirred slowly till a gel was obtained. Composition of gel was shown in Table **2**.

Table 2: Composition of carbopol gel formulations

S. No	Ingredients	Quantity (%w/w)
1.	Carbopol 980	1%
2.	Propylene glycol	5%
3.	Methyl paraben	0.1%
4.	Propyl paraben	0.03%
5.	Water	q.s.

In-vitro drug release studies through cellophane membrane

In-vitro drug release study of 5-FU from ethosomal gel formulation was studied using a Franz glass diffusion cell. The receptor compartment contained 30ml of phosphate buffer (pH 6.8) and was constantly stirred with a magnetic stirrer at 100rpm. The temperature was maintained at 37±1°C. Cellophane membrane was mounted between donor and receptor compartment. Ethosomal gel was applied to the membrane. Samples (1ml) were withdrawn through the sampling port of the diffusion cell at predetermined time intervals and immediately replenished equal volume of fresh phosphate buffer. Samples were analyzed by UV spectrophotometer at 265nm. Sink condition was maintained throughout the experiment. The experiment was conducted in triplicate.

Drug release kinetic modeling

The kinetics of ketoconazole release from ethosomal gels was determined using the release kinetics of drug release into various kinetic equations: zero order release kinetics, first order release kinetics, higuchi model and korsemeyer-peppas model.

RESULT AND DISCUSSION

Vesicle shape and surface morphology

Transmission electron microscopy was used for visualizing the particle size and surface morphology of ethosomes. Investigation of formulations by TEM indicated spherical structure of vesicles with a smooth surface. TEM images of ethosomal formulations of 5-FU were as shown in Figure **1**.

Particle size and size distribution

Particle size and size distribution are critical factors in performance of ethosomes. Particle size and size distribution can be determined using light scattering technique and also by SEM or TEM. From particle size distribution data, it was evident that in case of ethosomes of lecivia 90, mean particle diameter was 202.3 ± 3.4 nm and major portion of particles were in range of 105 to 342 nm, for ethosomes of lecivia 70, mean particle diameter was 202.3 ± 7.1 nm and major portion of particles were in range of 105 to 396 nm (Figure **2** and Table **3**). The result showed that formulations have narrow size distribution. The formulations had low polydispersity index (PDI) in the range of 0.37-0.41. Therefore, the PDI indicated uniformity of the formulations.

Percentage entrapment efficiency

The % entrapment efficiency of 5-fluorouracil in ethosomal formulations of EL70 and EL90 were found to be 88.7 ± 2.3 % and 87.9 ± 1.7 % respectively(Table **3** and Figure **3**). The observed results presented comparatively higher % EE of 5-fluorouracil in ethosomes of EL70 than EL90.

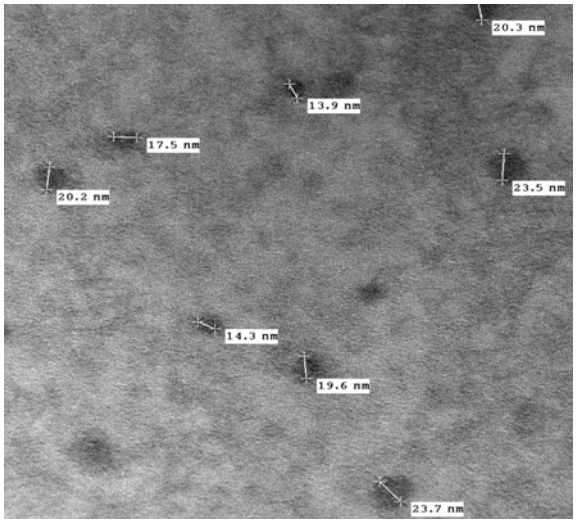
Zeta potential

The measurement of zeta potential allows predictions about the storage stability of ethosomal suspensions. In general, particle aggregation is less likely to occur for charged particles i.e. high zeta potential due to electric repulsion. A decrease in zeta potential i.e. electrostatic repulsion was considered as the cause for aggregetion process. Ethosomes of EL70 and EL90 were found to have zeta potential values of -26.9±0.98 and -1.35±0.35 mV(Table **3** and Figure **4**). It was obsedved that zeta potential of prepared vesicles has sufficient charge to inhibit aggregation of vesicles. Ethosomes have highest zeta value of -26.9 mV, indicating most stable vesicles.

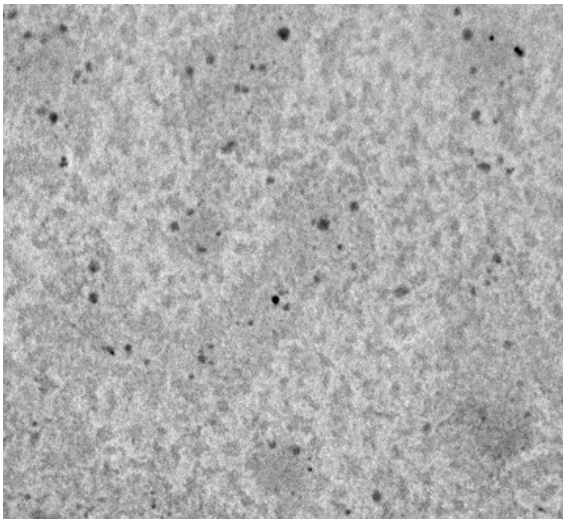
Table 3: Particle size, poly dispersity index, % entrapment efficiency, zeta potential of ethosomes of 5-fluorouracil

Formulation code	Particle Size (nm)	Polydispersity index	%Entrapment efficiency	Zeta potential (mV)
EL70	202.3±7.1	0.419±1.9	88.7±2.3	-26.9±0.98
EL90	202.3±3.4	0.370±1.2	87.9±1.7	-1.35±0.35

All values are represented as mean± standard deviation (n=3)



(a)



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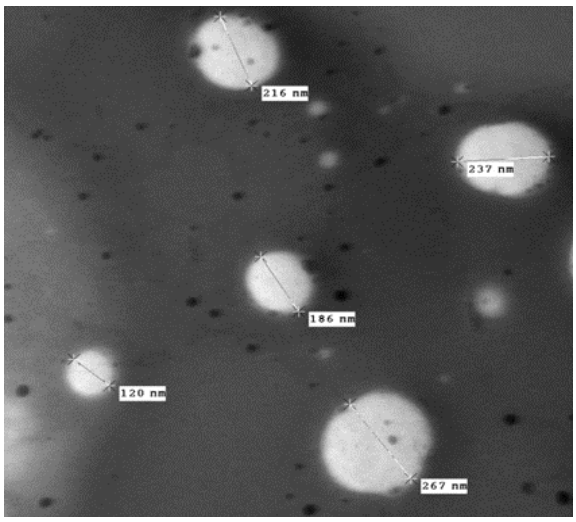
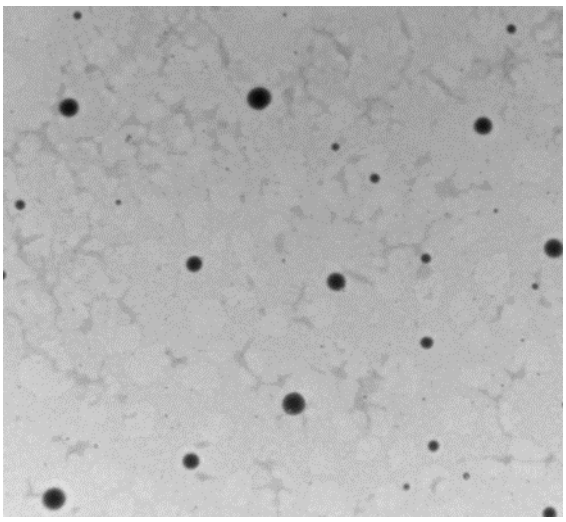
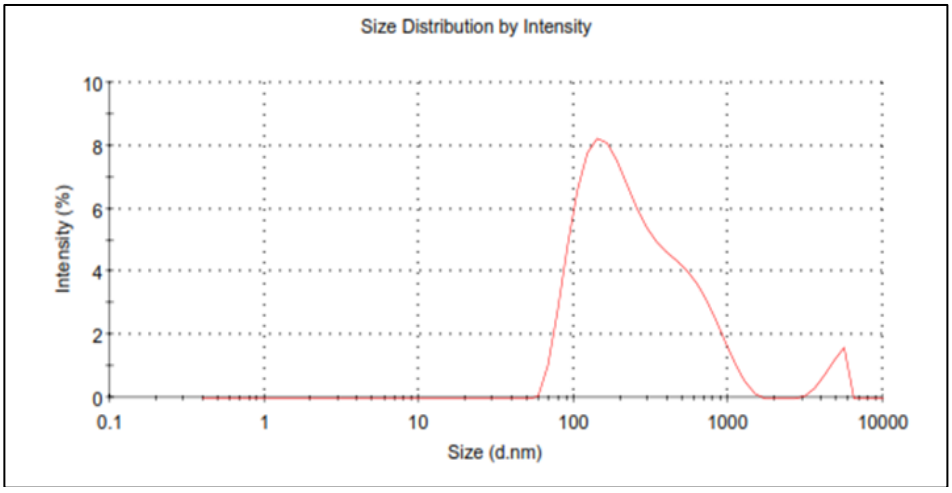


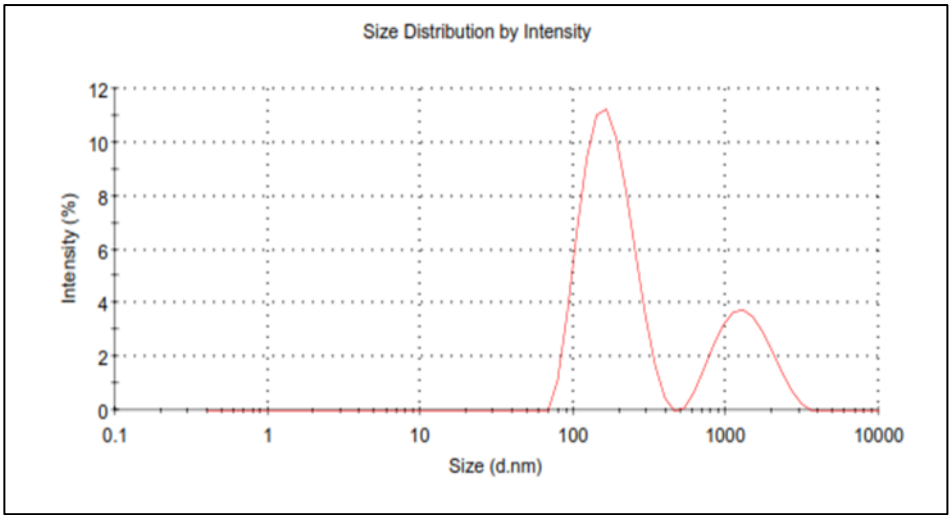


(c) **(d)**

Fig 1: Transmission electron photomicrographs
(a) and (b) ethosomes containing leciva70 (c) and (d) ethosomes containing leciva 90



(a)



(b)

Fig 2: Particle size distribution of ethosomal formulations (a) EL70 and (b) EL90

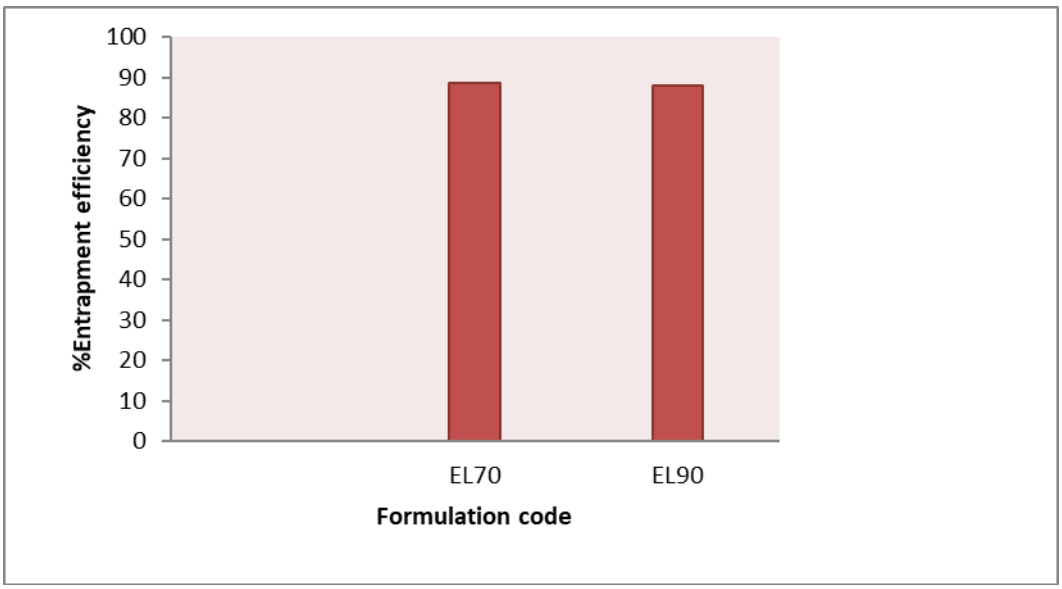
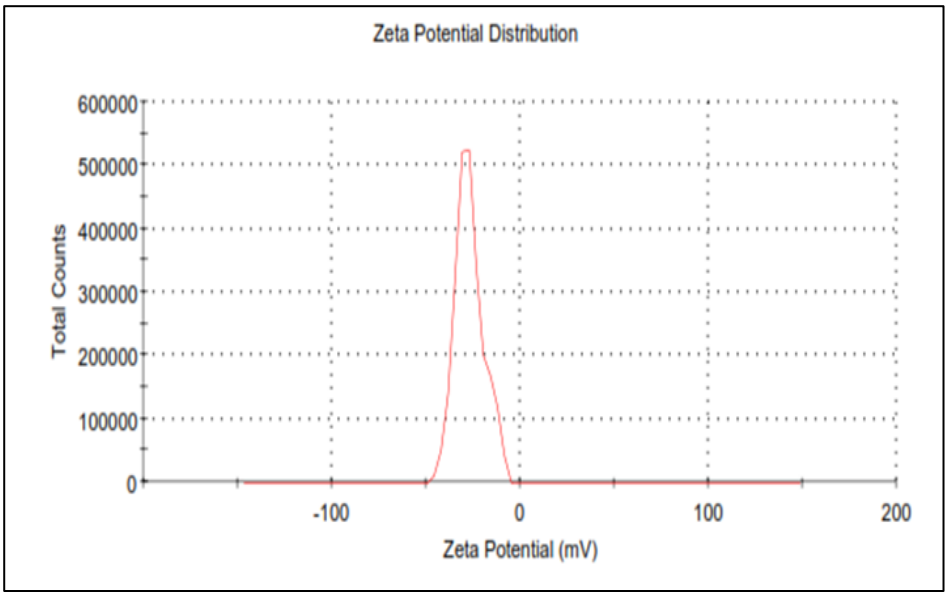
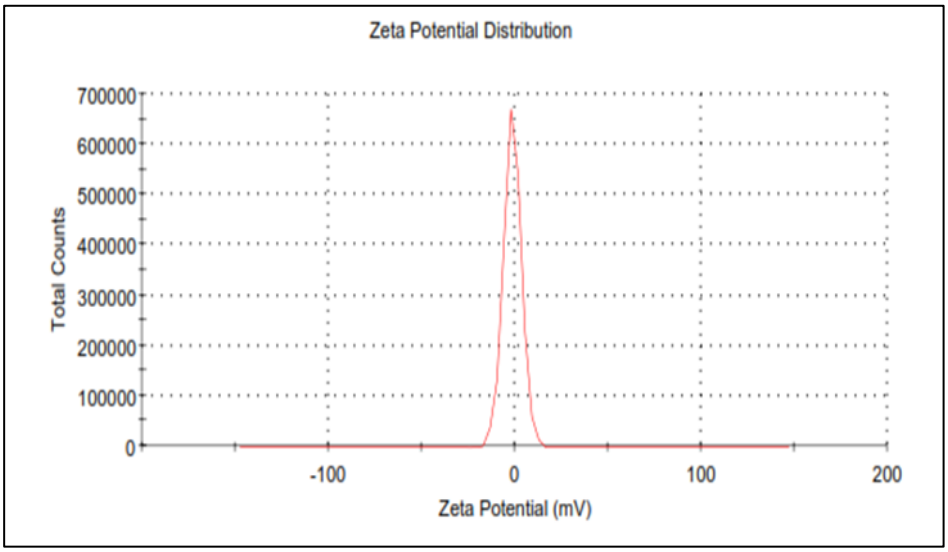


Fig 3: Entrapment efficiency of ethosomal formulations



(a)



(b)

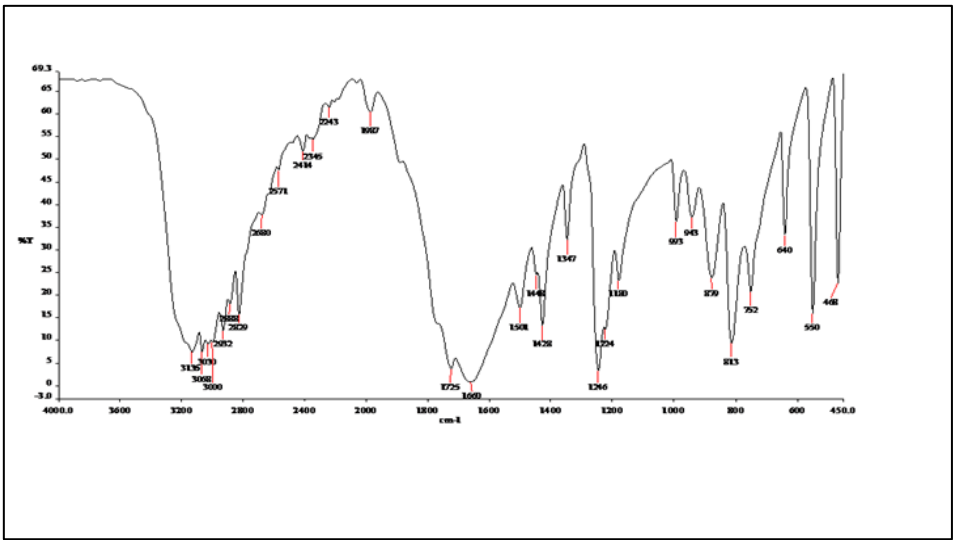
Fig 4: Zeta potential of ethosomal formulations (a) EL70 and (b) EL90

Fourier Transform Infrared Spectroscopy (FTIR)

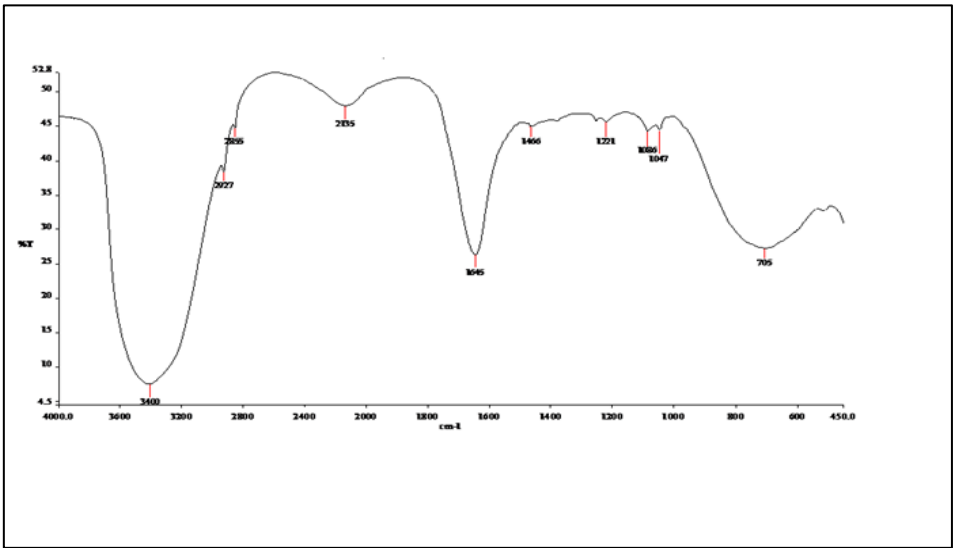
The possible interaction between drug and excipients were studied by IR spectroscopy. The FTIR spectra of pure drug 5-fluorouracil and prepared formulations EL70 and EL90 are as shown in Table **4** and Figure **5**. From IR data, it was observed that functionality of drug has remained unaffected, including intensities of the peak. This suggests that during development of formulation, excipients have not reacted with the drug to give rise to reactant products. So, there is no interaction between them which is in favor to keep for formulation.

Table 4: Major peaks observed in FTIR spectra of drug and formulations EL70 and EL90

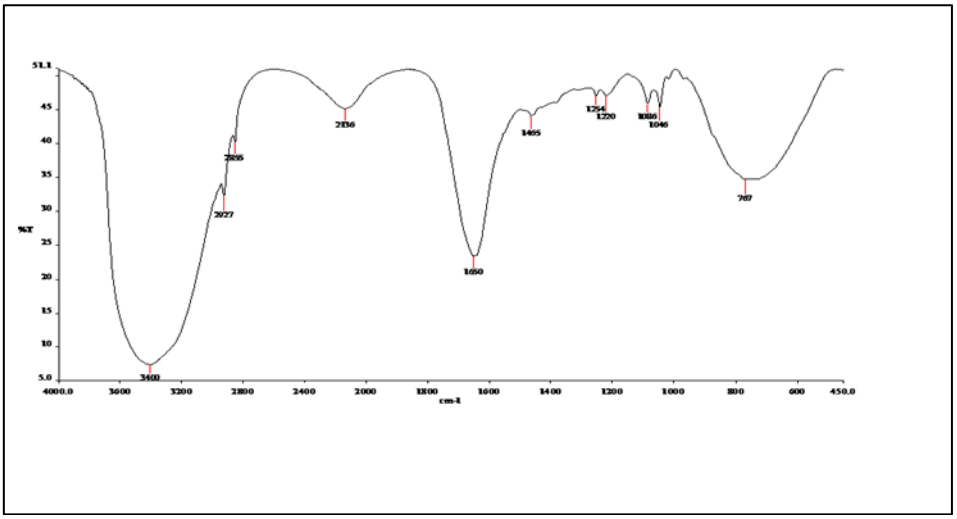
S. No.	Bands	Drug	EL70	EL90
1.	N-H stretch	3135cm^{-1}	3400cm^{-1}	3400cm^{-1}
2.	C-H stretch	2932cm^{-1} and 2829cm^{-1}	2927cm^{-1}and 2855 cm^{-1}	2927cm^{-1} and 2855cm^{-1}
3.	C=C stretch	1448cm^{-1}	1466cm^{-1}	1465cm^{-1}
4.	C-F stretch	1224cm^{-1}	1221cm^{-1}	1220cm^{-1}
5.	N-H bend	752cm^{-1}	705cm^{-1}	767cm^{-1}
6.	C=O stretch	1660cm^{-1}	1645cm^{-1}	1650cm^{-1}



(a)



(b)



(c)

Fig 5: FTIR spectra of drug (a) and ethosomal formulations (b) EL70 and (c) EL90

In-vitro drug release study through cellophane membrane

A comparative release profile of ethosomal gel formulations and PBS pH 6.8 drug solutions are illustrated in Figure **6**. Cellophane membrane was used that only allow the diffusion of drug in solution form while it withholds the lipid vesicles. Result revealed that % cumulative drug release from ethosomal formulations was found in the range from 66.22 ± 2.5 to 73.98 ± 3.8. Drug release from ethosomal gels was higher as compared to the plain drug solution.

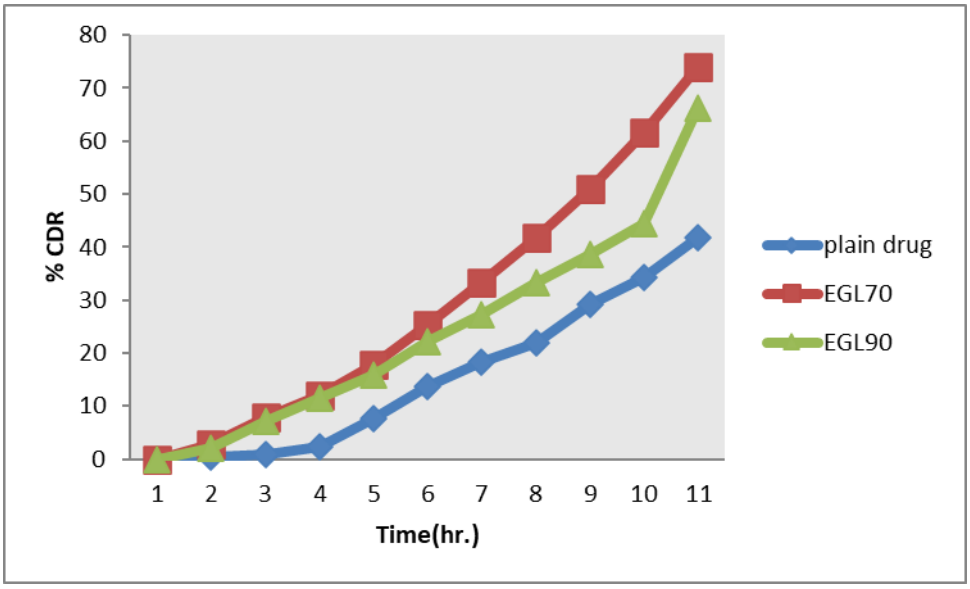


Fig 6: Release profile of 5-fluorouracil permeated across cellophane membrane from plain drug and ethosomal formulations

Drug release kinetics

Data obtained from *in vitro* drug release were fitted into the equations for zero order, first order, higuchi and korsemeyer-peppas models (Table **5**). The data interpretation was based on the value of resulting regression coefficients (r^2). The formulation EGL70 was best fitted for the zero-order kinetic equation as the formulation regression coefficient values predominates over the first order and higuchi kinetics.

Table 5: Permeation kinetics of 5-fluorouracil ethosomal formulations

Formulation code	Zero order model (r^2)	First order model (r^2)	Higuchi model (r^2)	Korsemeyer-peppas model (n)
EGL70	0.974	0.937	0.816	1.61
EGL90	0.942	0.837	0.794	1.54

CONCLUSION

Ethosomes, a novel vesicular carrier system was prepared successfully by cold method for prolonged as well as controlled release, improve the permeability of 5-fluorouracil, an anticancer drug across stratum corneum. The prepared formulation was characterized for various evolutionary parameters like morphology, vesicle size, entrapment efficiency, zeta potential and *in vitro* drug release study by using cellophane membrane. *In vitro* drug release study revealed that ethosomal formulations have been improved the drug release through cellophane membrane as compared to plain drug solution.

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