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Product Development of Beta-Sitosterol Ethosomes for the Treatment of Rheumatoid Arthiritis

Mehavarshini S[1],Sanjana Emily James[2],Arunkumar D[3],Monish Kumar S[4], Dr. H. Gayathri[5*]

[1,2,3,4,5]SRM College of Pharmacy, SRMIST, Kattankulathur campus, Tamil Nadu. India*

Corresponding Author: Dr.H.Gayathri[5]*
[5]SRM College of Pharmacy, SRMIST,Kattankulathur campus,Tamil Nadu, India.*
Email: [5]gayathrh@srmist.edu.in*

Abstract
Ethosomes are "non-invasivecarrier systems" currently in trend for theirnovelty and targeted drug delivery system.The aim of this pharmaceutical study is the product development and evaluation of Beta-sitosterolethosomes for the treatment of Rheumatoid Arthritis.The ethosomal treatments provide several benefits over the traditional treatments. The ethosomes were prepared using thin-film hydration methodfollowed by ultra-sonification method. Firstly, the drug Beta-sitosterol, phospholipid i.e., soya lecithin and cholesterol were dissolved in organic solvent ethanol. The organic solvent is evaporated using magnetic stirrer at 100 rpm to form thin lipid layer. Secondly, propylene glycol is added and stirred for 30 mins. Finally, the dried lipid layer is hydrated using a dispersion medium-water to form heterogenousethosomes. A bath sonicater is used to procure homogenous ethosomes mixture. The prepared ethosomes were evaluated or characterized by using Drug content, Entrapment efficiency, Particle size distribution, Zeta potential, *In-vitro* drug release, Scanning electron microscopy and Stability studies. The results of the evaluation study showed optimized ethosomal formulation F3 possessing Beta-sitosterolwith the highest drug content of 94.12%, entrapment efficiency of about 91.62% and having maximum in-vitro drug release of about 96% when compared with other formulations consisting of variable concentrations.Stability studies were initiated and it will be examined after 3 months.

Keywords: Ethosomes, Thin-film hydration method, Beta-sitosterol, Anti-inflammatory activity, Rheumatoid Arthritis

1. INTRODUCTION

The current and emerging approaches of novel carriers for the optimization of drug delivery has opened new avenues in the medical field. The advanced innovation in the topical route of drug delivery has several benefits over the traditional route of treatment.
Ethosomesare "Ethanolic liposomes". They are soft, malleable vesicles consistingof amphipathic phospholipids arranged inbilayers enclosing aqueous compartments.They are composed of phospholipids, ethanol (20-45% concentration), and waterthat enable drugs to reach the deep skinlayers and / or the systemic circulation. The new research area increases bioavailability and decreases the side effects of drug delivery systems.Ethosomes have better properties than liposomes, it eliminates gastrointestinal interferences, betterfirst pass metabolism of the drug, improved room temperature stabilityand encounters the barrier properties of the stratum corneum [1-3].
Beta-sitosterol is a type of chemical called a plant sterol.It belongs to the class of phytosterols and category stigmastanes. It's similar in structure to cholesterol and is found in fruits, vegetables, nuts, and seeds. It is a white, waxy powder with a characteristic odor, and is one of the components of the food additive [4,5]. Main function of Beta-sitosterol is reduction of cholesterol levels by limiting the amount of cholesterol that is able to enter the body. It is also used for the treatment of benign prostatic hyperplasia, heart disease, cancer, diabetes, hair loss and migraine [6]. Studies have shown that Beta-sitosterol interfere with multiple cell signaling pathways, including cell cycle, apoptosis, proliferation,

survival, invasion, angiogenesis, metastasis and inflammation [7]. However, the effect of Beta-sitosterol in Rheumatoid Arthritis remained largely unexplored. Studies show that Beta-sitosterol modulate macrophage polarization and attenuates rheumatoid inflammation in mice [8].
Rheumatoid Arthritis (RA) is a type of auto-immune disease that targets the synovial tissues of the joints. The immune system of the body destroys its own tissue, including joints. Early symptoms include swelling, heat, decreased joint function, and pain; later stages include varying degrees of deformity and joint stiffness, as well as bone deterioration and disability risk [9,10].RA affects about 1% of the global population, with women being affected more than males. RA usually appears between the ages of 30-60 in women, and slightly later in men [11].The risk factors for RA include both modifiable lifestyle-associated variables and non-modifiable features, such as genetics and sex [12].
The conventional treatment for Rheumatoid arthritisis Disease-Modifying Anti-Rheumatic Drugs (DMARDs), NSAIDS, Steroids and Biologics [13]. These conventional anti-inflammatory drugs have been unsuccessful in the complete treatment of RA. These have also been associated with unwanted side effects. This has led to research for new treatments for this disease.The purpose of this study is to assess the anti-inflammatory action of Beta-sitosterol drug using in vitro models for the treatment of Rheumatoid arthritis without the unwanted side effects.

2. MATERIALS AND METHODS

1. Drugs and Chemicals:

The drug Beta-sitosterol was received as a gift sample from Vital Herbs, Delhi, India. Soyalecithin from Delpha Drugs and Pharmaceuticals India, Cholesterol from S.D. Fine Chemicals Ltd., India, Ethanol from Alkem Laboratories Limited and Propylene Glycol fromSisco Research Laboratories Pvt. Ltd.

2. Instruments:

FTIR Spectrometer (Nicolet), Weighing Balance (Shimadzu AY220, Japan), Magnetic Stirrer(REMI), Sonicator (Quanta FEG), Centrifuge (REMI), UV Visible Spectrophotometer(Lab India Analytical UV 3092), Zeta Sizer (DTS Version 5.03, Malvern), Franz Diffusion Cell (OEM Manufactures India), Scanning Electron Microscopy (Quanta FEG)

3.Pre-Formulation Studies:

Compatibility study by FT-IR

The compatibility between the pure drug andexcipients were detected using FTIR Spectroscopy by checking for anu shifts or peaks in the spectra. The spectra were recorded over the wave number of 4500 to 500cm^{-1} [14].

Development of calibration curve

The standard stock solution of Beta-Sitosterol was prepared by dissolving 100mg of Beta-sitosterol in 50 ml of distilled water and sonicated for 15 mins. The final volume of solution made upto 100ml with same solvent to get stock solution containing 100µg/ml. The stock solution series of concentrations 2, 4, 6, 8, 10 µg/ml were taken and made upto 10 ml with distilled water. The absorbance was measured to plot the calibration curve [15].

4. Preparation of Ethosomes:

The ethosomes loaded with Beta-sitosterol were formulated using thin film hydration method followed by ultra-sonification method. Beta-sitosterol, phospholipid i.e., soya lecithin and cholesterol were used to prepare ethosomes. In 10ml of ethanol, weighed amount of drug, soya lecithin and cholesterol were dissolved and then put in a clean and dry bottomflask. The organic solvent was carefully evaporated using a magnetic stirrer at 100 rpm at room temperature to form a lipid film on the flask wall. To this 5ml propylene glycol was added, followed by water and stirred for 30 mins. A

bath sonicator is then used for 10 mins to sonicate the ethosomes for homogenization of vesicles [16,17]. The compositions of various ethosomal formulations are represented in table 1.

Table 1: Different Compositions ofEthosomes Formulation

Formulation Code	Beta-sitosterol (mg)	L-Soyalecithin (mg)	Cholesterol (mg)	Ethanol (ml)	Propylene Glycol (ml)	Water (q.s.)
F1	60	250	5	10	5	q.s.
F2	60	200	5	10	5	q.s.
F3	60	150	5	10	5	q.s.
F4	60	100	5	10	5	q.s.
F5	60	50	5	10	5	q.s.
F6	60	25	5	10	5	q.s.

5. Evaluation of Ethosomes:

Percentage drug content

The drug content was evaluated by taking 1g of the ethosomal formulation and diluting it with distilled water in a 100ml volumetric flask. The absorbance was measured using UV Spectrophotometer at 210 nm to calculate the drug content [18].

Concentration of drug (µg/ml) = (Slope × Absorbance) ± Intercept

Entrapment efficiency

Entrapment efficiency was evaluated using the unentrapped drug in the supernatant. The drug loaded ethosomes dispersion was added to the centrifugation tube and centrifuged at 10000 rpm for20 mins. The drug entrapped ethosomes settle at the bottom of the tube. The entrapment efficiency was calculated by measuring the absorbance of the supernatant in UV Spectrophotometer at 210 nmand amount of drug was calculated by using regression equation which was obtained from the standard plot [19].

$$\textit{Entrapment efficiency (\%)} = \frac{\textit{Total amount of drug} - \textit{Unentrapped drug} \times 100}{\textit{Total amount of drug}}$$

$$\textit{Amount of drug} = \frac{(\textit{Concentration of drug} \times \textit{Dissolution bath volume} \times \textit{Dilution factor})}{1000}$$

Particle size and zeta potential

The particle size and zeta potential of the vesicles were determined by photon correlation spectroscopy (PCS) using Malvern zeta sizer at a fixed angle of 90° at 25 °C using water as a dispersant for measurement of particle size and zeta potential [20,21].

In-vitro drug release studies

The *in-vitro* drug release studies were done using a modified Franz diffusion cell.The cellophane semi-permeable membrane (Molecular weight cut off 12000–14000, HI Media Ltd, Mumbai, India) was soaked in glycerin for 12 hours. The cellophane membrane was mounted on a diffusion cell assembly with an effective permeation area and receptor cell volume of 2.4 cm and 200 ml, respectively. The receptor compartment consisted of a 30 ml phosphate buffers at pH 7.4 as the receptor fluid agitated at 100 rpm and was maintained at 37 ± 0.5°C throughout the experiments. Between the donor and receptor chambers, a dialysis membrane was attached. The prepared formulation was applied to the membrane in the donor compartment. Samples of 5 ml were

withdrawn at different time intervals and replaced immediately with an equal volume of fresh phosphate buffer at pH 7.4.It was then analyzedspectrophotometrically at 210nm. The cumulative amount that permeated across the cellophane membrane was calculated and plotted against time [22].

Visualization by scanning electron microscopy (SEM)

Scanning electron microscopy was used for determining the surface morphology of the formulated ethosomes. The ethosomal suspension was diluted as needed. A few drops of the suspension were dropped onto the grid and left to dry. Images were captured on a scanning electron microscope (magnification: 60x, accelerating voltage: 12.0kV, and temperature: 252°C) after the materials were fully dried [23].

Stability studies

The stability studies were carried out according to percentage entrapment efficiency and drug content at 25 ± 2°C for a period of 30 days. The drug content was evaluated regularly for any change in entrapment efficiency of the formulations [24,25].

6. RESULTS AND DISCUSSION

1. Pre-Formulation Studies:

Compatibility study by FT-IR

IR spectrophotometry has been employed as a useful tool to identify the drug excipient interaction. The figures illustrate the IR spectrum of Beta-sitosterol, the physical mixture with
Soya lecithin and cholesterol. The IR spectra indicate there were no interactions between the drug and excipients as there was no major shifting, appearance or missing of peaks observed.

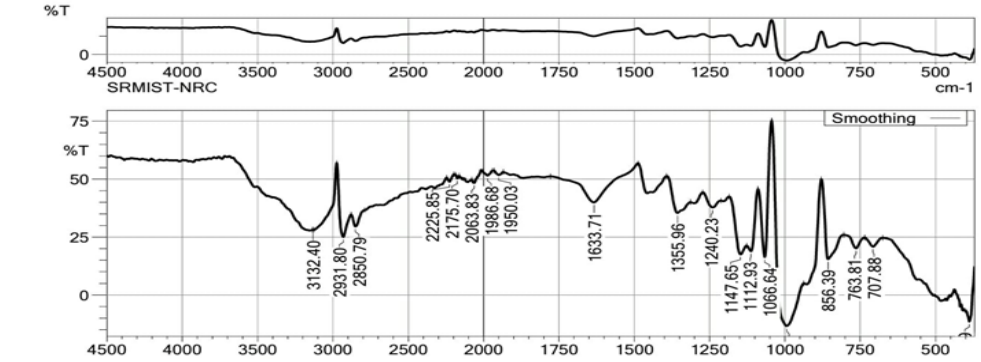


Figure 1: FTIR Spectra of Beta-sitosterol Pure Drug

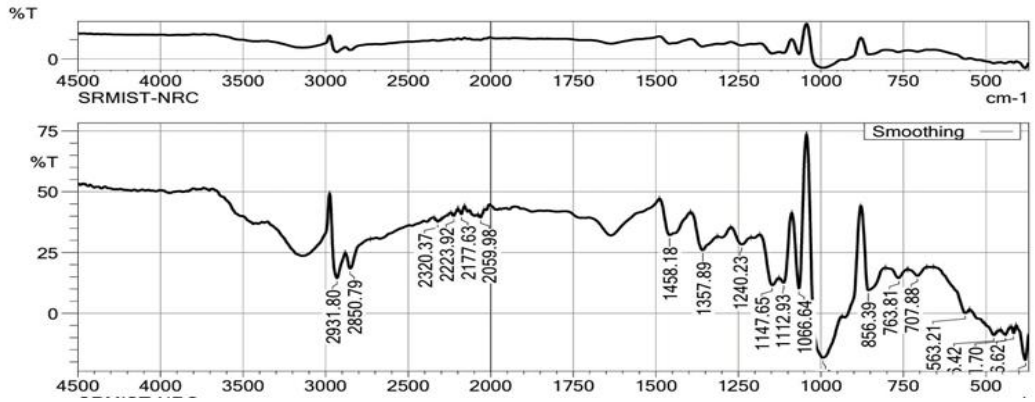


Figure 2: FTIR Spectra of Beta-sitosterol with excipients soya lecithin, cholesterol

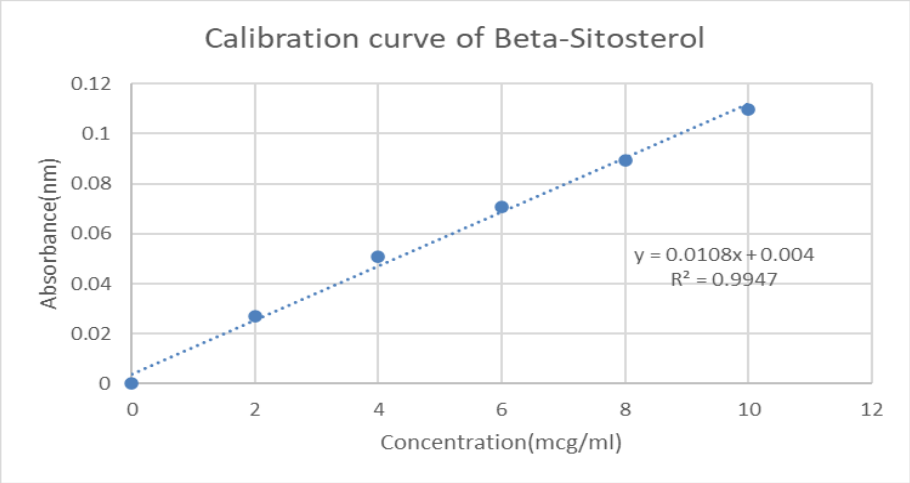
Development of calibration curve

The maximum absorption of Beta-Sitosterol in distilled water was found to occur at a wavelength of 210nm and all obtained values are given in the Table 2. At a given concentration the standard curve obtained for the drug Beta-Sitosterol obeys Beer's law. The calibration graph for the drug was created by plotting absorbance vs. concentration (µg/mL) at 210nm. The relationship between absorbance and concentration is found to be linear, having a regression coefficient value of 0.9947 when subjected to regression analysis. Linear relationship was derived between both the variables, absorbance versus concentration, y = 0.0108x + 0.004.

Table 2: Calibration curve

S.NO	CONCENTRATION (mcg/ml)	ABSORBANCE 210nm
1.	0	0
2.	2	0.027
3.	4	0.051
4.	6	0.0709
5.	8	0.0893
6.	10	0.1097

Figure 3: Calibration graph of Beta-sitosterol



1. **Evaluation of Ethosomes:**

Percentage drug content

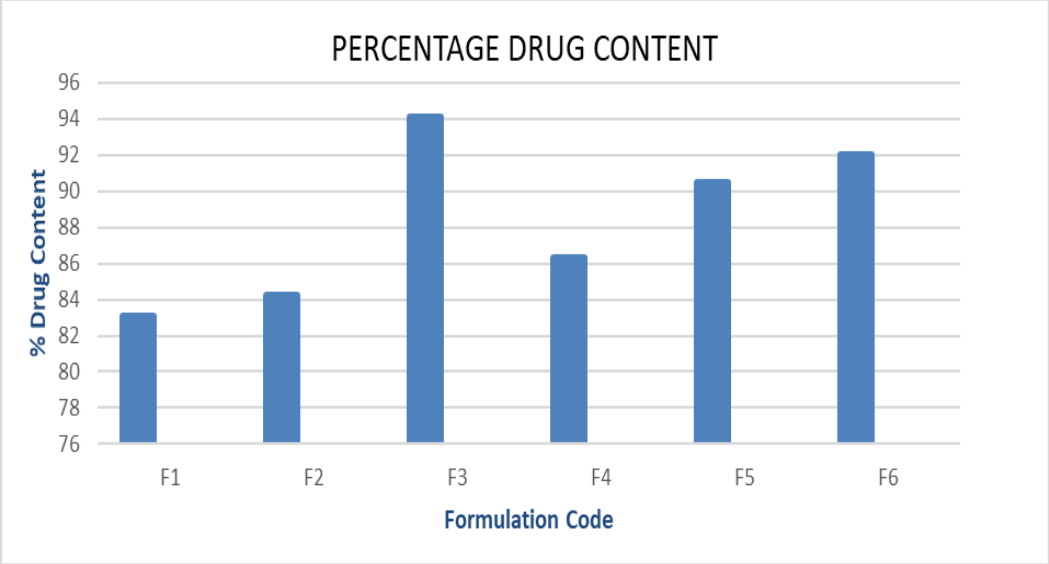
The drug content was calculated for Beta-sitosterol by using UV Visible spectrophotometer at 210 nm and all the obtained values are given Table 3.

Table 3: Concentration of drug

S.NO	FORMULATION CODE	CONCENTRATION (mcg/ml)
1.	F1	83.06
2.	F2	84.21
3.	**F3**	**94.12**
4.	F4	86.35
5.	F5	90.44

6.	F6	92.01

Figure 4: Percentage drug content



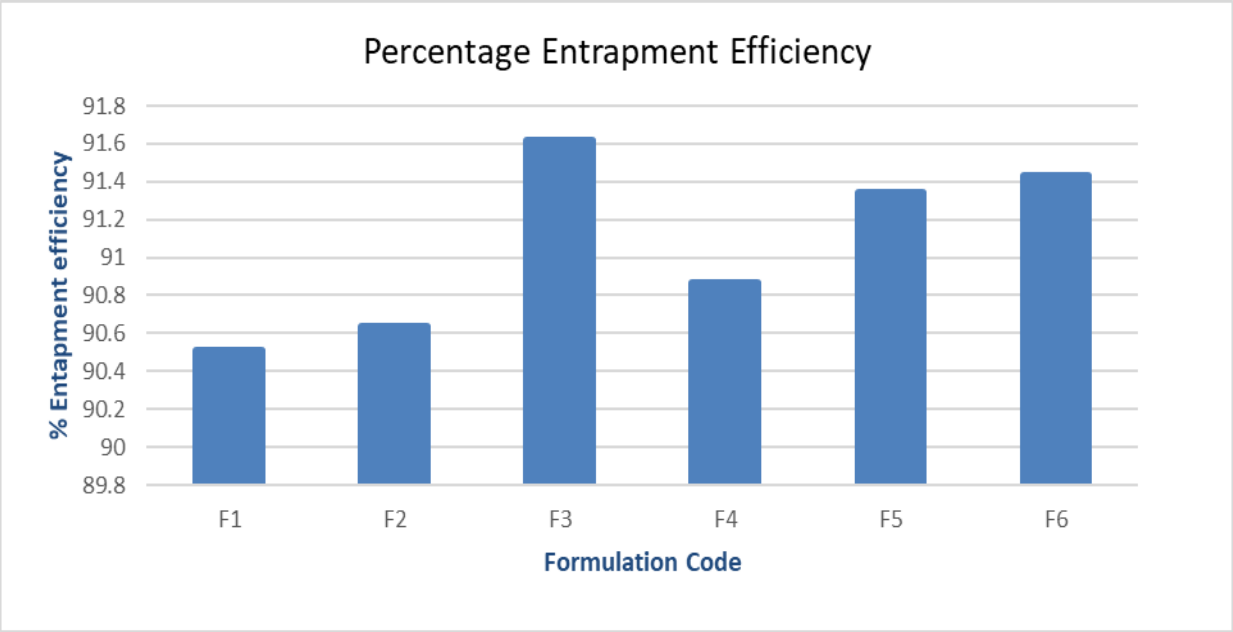
Entrapment efficiency

The entrapment efficiency was calculated for Beta-Sitosterol. The obtained values are given in Table 4. The maximum entrapped efficiency was observed with the batch F3 with about 91.62%. This was comparatively higher than other batches.

Table 4: Entrapment efficiency of Beta-sitosterol

S.NO	FORMULATION CODE	ENTRAPMENT EFFICIENCY (%)
1.	F1	90.51
2.	F2	90.64
3.	**F3**	**91.62**
4.	F4	90.87
5.	F5	91.34
6.	F6	91.43

Figure 5: Entrapment efficiency of Beta-sitosterol



Particle size and zeta potential

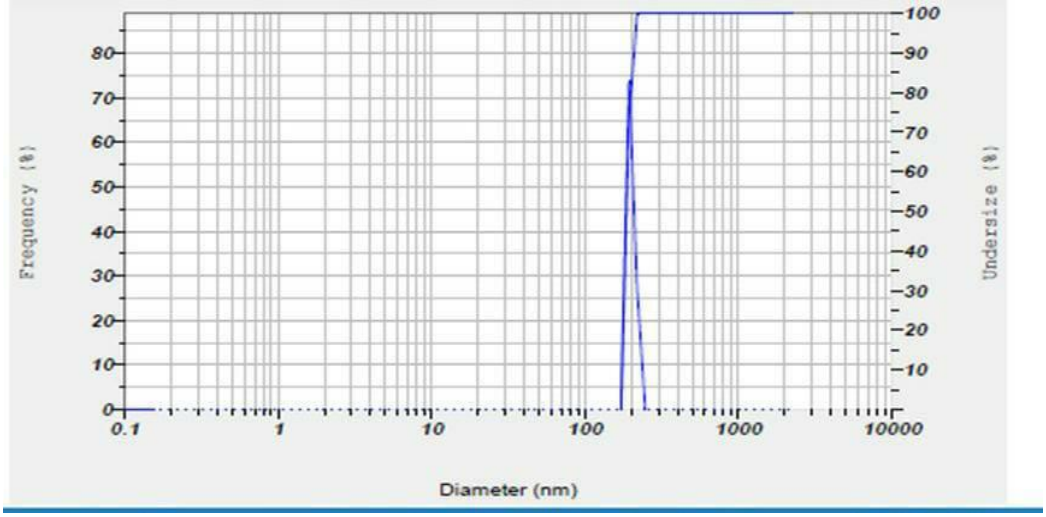
The zeta potential and particle size of the F3 formulation were determined using Zetasizer (DTS Version 5.03, Malvern) using light scattering method. The mean vesicle diameter was found to be 1299.8 nm. The zeta potential was found to be -0.1 mV.

Table 5: Particle size and zeta-potential of F3 formulation

FORMULATION	ZETA-AVERAGE SIZE	PDI
F3	1299.8 nm	0.694

The normal size of the vesicles and its distribution is confirmed by the obtained size distribution curve.

Figure 6: Size distribution curve



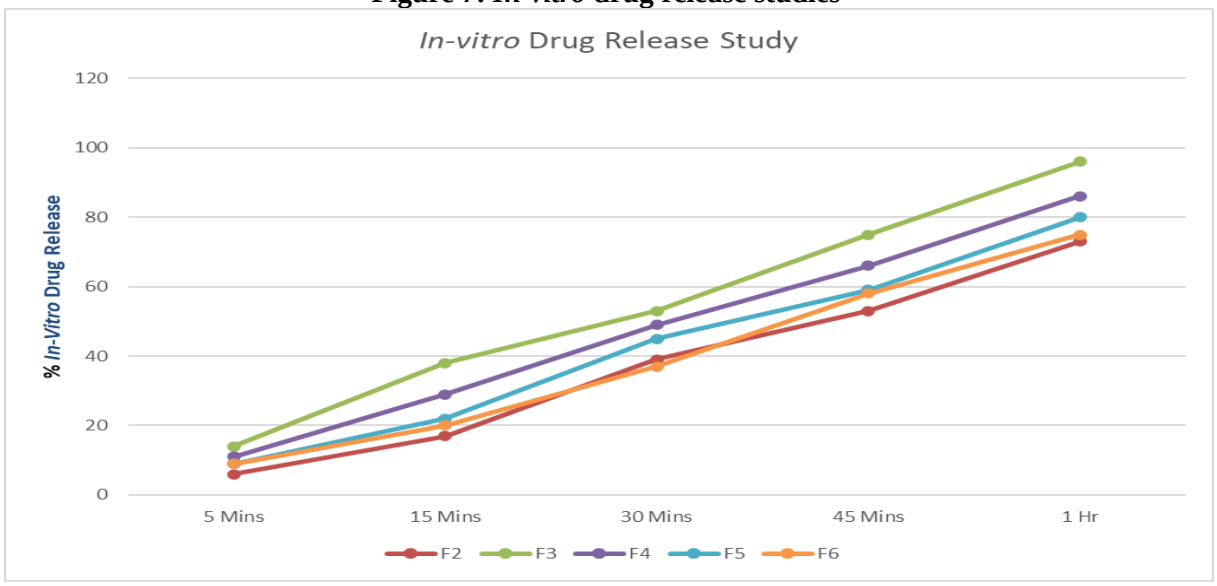
***In-vitro* drug release studies**

In-vitro release studies by using Franz diffusion cell was performed for all the formulations at 37°C. A graph of percentage cumulative drug release vs time was plotted. From the results it was observed that all the batches showed a good in-vitro release pattern. But the batch F3 showed a maximum release of 96% at the end of 6 hours.

Table 6*: In-vitro* cumulative percentage drug release

S.NO	TIME	CUMULATIVE % RELEASE					
		F1	F2	F3	F4	F5	F6
1	5 mins	5±0.74	6±1.3	14±1.6	11±0.5	9±0.3	9±0.42
2	15 mins	17±0.26	17±0.36	38±1.1	29±0.6	22±0.34	20±0.17
3	30 mins	38±0.7	39±1.3	53±0.4	49±0.87	45±0.27	37±0.32
4	45 mins	51±0.42	53±0.11	75±0.17	66±0.37	59±0.29	58±0.2
5	1 hr	67±0.11	73±0.23	96±0.43	86±0.33	80±1.1	75±0.2

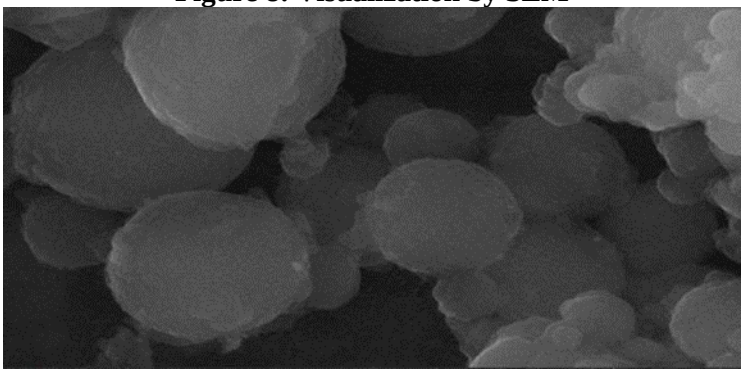
Figure 7: *In-vitro* drug release studies



Visualization by scanning electron microscopy (SEM)

Spherical droplets in the nanometres level were seen in the F3 micrographs. The results demonstrate that the particles were spherical in form and that no drug crystals were identified within them. Some particles morphology that deviates from sphericity may be caused by lipid modification during the sample treatment procedure. Furthermore, the particle form is determined by the lipid purity.

Figure 8: Visualization by SEM



Stability studies

The stability studies were initiated. All the parameters will be examined after 3 months, 6 months, 9 months and upto 1 year.

1. CONCLUSION

The Beta-sitosterolethosomes for the treatment of Rheumatoid arthritis was prepared and characterized. The optimized ethosomal formulation F3 possessing Beta-Sitosterol showed promising results having highest entrapment efficiency of about 91.62% and having maximum in-vitro drug release of about 96% when compared with other formulations consisting of variable concentrations. Stability studies were initiated and it will be examined after 3 months.

Therefore, it is concluded from the study that the formulation was safe and efficient carriers of the drug Beta-sitosterol for the treatment of Rheumatoid arthritis.

Future Scope

The F3 formulation with Beta-sitosterol loaded ethosomes shows the best effect on anti-inflammatory activity for arthritis. The formulation is used to prepare gel using Carbopol. The gel will be evaluated for pH, organoleptic characteristics, washability, spreadability, extrudability, and storage stability. The most effective gel formulation will be selected for further animal testing.

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