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Formulation of Coffee Bean Extract (Chlorogenic Acid) Solid Lipid Nanoparticles for Lymphatic Uptake on Oral Administration

Dr. Mangesh R Bhalekar *[1], Vishal Raskar[2]

[1, 2] AISSMS College of Pharmacy, SavitribaiPhule Pune University, Pune, India-411001

ABSTRACT

Coffee bean extract (Chlorogenic Acid), Disease Modifying Anti-Rheumatoid Drug (DMARD) agent in the treatment of Rheumatoid Arthritis is believed to inhibit the production of TNF-α by blocking the cell associated conversion of TNF Precursor to mature proteins this, halting the proliferation of synovitis.CGA inhibit the proliferation of the fibroblast-like synoviocyte cell line (RSC-364), stimulated by interleukin (IL)-6, through inducing apoptosis. CGA inhibit the inflammatory proliferation of RSC-364 cells mediated by IL-6 through inducing apoptosis. CGA was also able to suppress the expression levels of key molecules in the JAK/STAT and NF-κB signaling pathways, and inhibit the activation of these signaling pathways in the inflammatory response through IL-6-mediated signaling, thereby resulting in the inhibition of the inflammatory proliferation of synoviocytes. The aim of the present study was to formulate and evaluate coffee bean extract (chlorogenic acid) solid lipid nanoparticles using a positive charge on it by means of enabling lymphatic uptake. Coffee bean extract (chlorogenic acid) solid lipid nanoparticles were prepared by melt emulsification-high pressure homogenization method and the particle size, PDI and % entrapment efficiency was found to be 210nm, 0.455 and 91.18% .ex vivo endocytic uptake studies revealed engrossment of endocytic pathways in the uptake of solid lipid nanoparticles from intestine.

Keywords: Coffee bean extract (Chlorogenic Acid), Glyceryl monostearate, Solid Lipid Nanoparticles, Lymphatic Uptake

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***Address for Correspondence:**

Dr. Mangesh R Bhalekar, Professor, AISSMS College of Pharmacy, Savitribai Phule Pune University, Pune-411001. India.

Abbreviations: CGA-ChlorogenicAcid, GMS-Glyceryl monostearate, DMARD- Disease Modifying Anti-Rheumatoid Drug, SLN-Solid Lipid Nanoparticles.

1. INTRODUCTION

Coffee bean extract (Chlorogenic Acid), has been reported to serve as Disease Modifying Anti-Rheumatoid Drug (DMARD). Chlorogenic acid given orally in a dose of 40mg/Kg is reported to inhibit the production of TNF-α by blocking the cell associated conversion of TNF Precursor to mature proteins thus halting the proliferation of synovitis [1,2].

Solid lipid nanoparticles (SLN) are colloidal drug carriers and have particle size ranging from 50 nm to 1000 nm and its advantages include biocompatibility, stability and protects drug against chemical degradation[5]. SLN are reported to have ability to enter lymphatic circulation when administered orally through Peyer's patch[6,7],the factors such as particle size, zeta potential on particles are known to affect the uptake[6]

The present work attempts to formulate chlorogenic acid in solid lipid nanoparticulate carrier by using principal of design of experiment (DOE) and demonstrate its lymphatic uptake by *ex vivo* everted rat gut sac model.

2. MATERIAL AND METHODS:

2.1. Materials

Coffee bean extract was purchased from shamantak enterprises (pune, india). Glyceryl mono stearate (analab fine chemicals),chlorogenic acid (sigma aldrich) purchased locally. All the other chemicals and reagents were of analytical grade and procured from local sources.

2.2 Methods

2.2.1 Characterization of Coffee bean extract:

2.2.1.1 Standard calibration curve of chlorogenic acid by UV-Visible spectrophotometry

Solution of chlorogenic acid were prepared in concentration range of 5 to 30µg/ml in ethanol. The absorbance of resulting solutions was measured at 324 nm using double beam UV-Visible Spectrophotometer (LABINDIA UV 3000) against ethanol as blank [10].

2.2.1.2 Selection of lipid using solubility parameter:[11,12]

Selection of lipid for preparation of solid lipid nanoparticles was done on the basis of difference in the solubility parameter of drug and various lipids. The value of solubility parameter of the drug and each lipid was calculated on the basis of their Molecular Structure. The Molecular structure is having different groups as well as bonds and so ΔE_υ and Vm values are specific for each group. The $\boldsymbol{\delta}$ for each molecular structure was calculated by using the formula given below, and then the difference between the Solubility parameter of drug and the lipid was noted.

$$\boldsymbol{\delta = (\Delta E\upsilon / Vm)^{0.5}} \quad \text{..... Equation (1)}$$

where, ΔE_υ -the energy of vaporization at a given temperature and

Vm -the corresponding molar volume which is calculated from the known values of molecular weight and density.

2.2.1.3 Selection of Surfactant System:[13]

A mixture of Coffee bean extract (Chlorogenic Acid) and GMS in ratio 1:3 was melted at 70°C. Aqueous phase was prepared by dissolving separately aqueous phase surfactants Tween 80 (4%), sodium lauryl sulphate (4%) and poloxamer 188 (4%) into distilled water at 70°C. Two phases were mixed at the same temperature followed by stirring under an over-head stirrer at 800 rpm for 15 minutes to obtain a uniform emulsion, which was further subjected to high pressure homogenization at homogenization pressure (400 bars) and homogenization cycles (3). The resultant solid lipid nanoparticles were evaluated for particle size and PDI. The surfactant system was chosen depending upon the average diameter of the globules produced and Poly Dispersity Index (PDI) of the resultant emulsion upon employing the surfactant system. (Table 1).

Table 1: Trial combinations of Surfactant used for preparation of Solid Lipid Nanoparticles

Batches	Water Phase Surfactant (2%)
T1	Sodium Lauryl Sulphate
T2	Poloxamer 188
T3	Tween 80

2.2.1.4 Drug Excipient Compatibility Studies:

Compatibility of Coffee bean extract (Chlorogenic Acid) with GMS and Tween 80 was checked in physical mixtures (1:1 ratio) stored at 40^0C for 30 days . Fourier transform- infrared spectroscopic analysis (FT-IR) was carried out in Shimadzu-IR Spectrophotometer. Samples were dispersed in KBr and compressed into pellet by application of pressure. The canning range was 4000-400 cm^{-1} and resolution was 1cm^{-1}.

2.2.2 Optimization and formulation of Coffee bean extract SLN:

The Coffee bean extract was added to chosen lipid at 70°C. Aqueous phase was prepared by dissolving surfactant into distilled water at 70°C. Two phases were mixed at the same temperature followed by stirring under an over-head stirrer at 800 rpm for 15 minutes to obtain a uniform emulsion. This emulsion was further subjected to high pressure homogenization, the process parameters such as surfactant (Tween 80) concentration (2%, 4%, 6%), homogenization pressure (400,800,1200 bars) and homogenization cycles (3, 6, 9) were optimized using a 3 factor 3 level Box Behnken Design to obtain SLN of smallest particle size and highest % entrapment.

The layout of the experimental design is shown in table 2 and 3.

Table 2: Factor combination and respons parameters for the Box Behnken Design

Sr. No.	Factors				Responses
1	Concentration of Aqueous Surfactant (Tween 80) (%)	-1	0	+1	Percentage Entrapment Efficiency (%)
2	Homogenisation pressure (Bar)	-1	0	+1	Particle Size (nm)
3	homogenization cycles (No)	-1	0	+1	

Table 3: Translation of experimental conditions into physical units for preparation of Coffee bean extract(Chlorogenic Acid) SLN.

Sr. No.	Factor 1(A): Aqueous Phase Surfactant (Tween 80) (%)	Factor 2 (B):homogenization pressure (bar)	Factor 3 (C): homogenization cycles (No)
-1	2	400	3
0	4	800	6
+1	6	1200	9

A blank SLN batch (excluding coffee bean extract) as per optimized formula was also prepared similarly.

2.2.3 Characterization of Coffee bean extract SLN:

2.2.3.1 Determination of Particle size:

The particle size analysis of the prepared SLN dispersions was performed using Malvern Zetasizer ZS 90 (Malvern Instruments, Worcestershire, UK), utilizing laser diffraction with beam length 2.40 mm, range lens of 300 RF mm, and at 14.4% obscuration. The sample was diluted with distilled water prior to the analysis. The mean diameter and the poly dispersity index of each batch was recorded.

2.2.3.2 Determination of Entrapment Efficiency:

The SLN dispersion was subjected to centrifugation at 20,000 rpm for 15 minutes and the pellets of sediment Solid Lipid Nanoparticle was separated from the clear supernatant liquid. The pellet was analysed for the chlorogenic acid content spectrophotometrically at 324 nm using ethanol as a solvent, similarly the supernatant was also analysed for unentrapped Coffee bean extract (Chlorogenic Acid). EE was calculated according to the equation 2.

$$EE\% = \frac{The\ amount\ of\ entrapped\ drug\ in\ SLN}{The\ amount\ of\ drug\ in\ SLN\ and\ free\ drug\ in\ dipersion} X\ 100 \text{..... Equation (2)}$$

2.2.3.3 Differential Scanning Calorimetry (DSC):

Differential scanning calorimetry (DSC) studies were carried out on Coffee bean extract (Chlorogenic Acid), and its physical mixture with GMS using Mettler Toledo DSC 823 *e software (Columbia, USA). Samples were accurately weighed and heated in sealed aluminium pans at a rate 10°C/min between 30- 300°C temperature rang under nitrogen atmosphere at flow rate of 40ml/minute.Empty aluminium pan was used as a reference.

2.2.4 *Ex vivo* endocytic uptake study[15]:

It has been seen that there are multiple methods reported for determination of uptake of lipid nanoparticles from an intestine. In order to study the uptake of Coffee bean extract SLN across rat intestine, *ex vivo* endocytic uptake study using everted rat intestine model was done. Segments of small intestine of sacrificed rat were procured, cleaned and everted using a glass rod.[16,17] One end of the intestinal segment was sealed using a silk suture, while from the other open end, 1 ml of phosphate buffer of pH 6.8 was added inside and sealed using silk suture. The resultant sac was further incubated in a dispersion containing Coffee bean extract SLN. Coffee bean extract (Chlorogenic Acid) concentration in the phosphate buffer was estimated using UV method.

The uptake mechanism of the SLN by the intestinal cells was evaluated in detail, everted gut sacs as described above were also incubated with specific endocytic inhibitors like chlorpromazine (concentration of 10 µg/ml) and nystatin (concentration of 25µg/ml) at 37 °C for 30mins and in Coffee bean extract SLN dispersions at 4°C. After 30mins of incubation the phosphate buffer from the intestinal sacs were carefully collected in test tubes and subjected to spectrophotometric analysis.

3. RESULTS AND DISCUSSION

3.1.1 Standard calibration curve of chlorogenic acid by UV-Visible Spectrophotometry:

Three absorption maxima viz 221, 253 and 324 nm for chlorogenic acid are reported in literature [18]. However the linearity was seen at 324 nm; hence all analytical measurements were done at 324 nm.

Ethanolic solution of chlorogenic acid obeyed Beer's law between concentration range of 5-30µg/ml. the equation of line and R^2 was found to be y = 0.036x + 0.030 and R^2= 0.993 respectively figure 1.

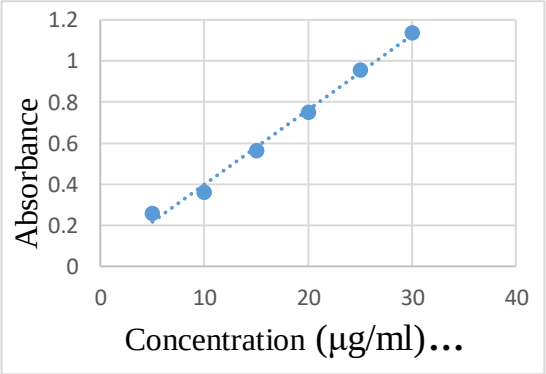

Figure 1: Calibration curve of chlorogenic acid

3.1.2 Selection of Lipid based on Solubility Parameter:[11,12]

The solubility parameter of a liquid δ, defined as the square root of the cohesive energy density is a quantity which, in conjunction with suitable theory, allows one to estimate several thermodynamic properties of solutions. The cohesive energy density itself is defined as the ratio of the energy of vaporization, ΔE_υ to the molar volume both referred to the same temperature. Solubility parameters are widely used to describe the cohesive forces within materials and have been used to describe many physical properties of a material and predict interactions between materials. Fedor's group contribution method of estimating solubility parameter, also based on group additive constants is believed to be superior to Small's method for two reasons: 1) the contribution of a much larger number of functional groups have been evaluated, and 2) the method requires only a knowledge of the structural formula of the compound.

The combination of drug and lipid with least difference of solubility parameter gives highest solubility hence GMS was selected on this basis as the lipid carrier. Table 4

Table 4: Solubility Parameter of Coffee bean extract(Chlorogenic Acid) and various lipids together with their differences.

Sr. No.	Drug/ Lipid Name	Solubility Parameter ($MPa^{1/2}$)	Difference with Coffee bean extract(Chlorogenic Acid) (Δδ)
1	Chlorogenic Acid	25.56	--
2	Compritol 888 ATO	21.26	4.3
3	Stearic Acid	18.66	6.9
4	Precirol ATO 5	21.63	3.93
5	Emulcire 61	19.27	6.29
6	GMS	22.28	3.28

3.1.3 Selection of Surfactant System:[13]

SLN containing sodium lauryl Sulphate (SLS) produced substantially smaller particle size 240.25 nm, but owing to high poly dispersity index (0.528) and associated instability, it was eliminated as a choice.Other surfactant such as poloxamer 188 had smaller particle size 208.73 nm and also low poly dispersity index (0.480) but due to its gelation on storage it was also not considered for further study. Tween 80 resulted in particle size of 220.5 nm and PDI of 0.455 and hence this was chosen to carry out the study in Table 5.

Table 5: Represents various findings of different amalgamations of lipid and water surfactant.

Batches	Solid Lipid (GMS) (%)	Water Phase Surfactant (Tween 80) (%)	Mean *Particle Size (nm)	*PDI	Observation
B1	GMS	Sodium Lauryl Sulphate	240.25	0.528	Higher Particle Size
B2		Poloxamer 188	208.73	0.480	Has Caused Gelation on storage
B3		Tween 80	220.5	0.455	Stable on Storage

*n=3 i.e. average of three readings

Tween 80 as a long chain surfactant provides aqueous phase stability to the emulsion formed thus leading to the formation of an emulsion system with low particle size and PDI.

3.1.4 Drug Excipient Compatibility Studies:

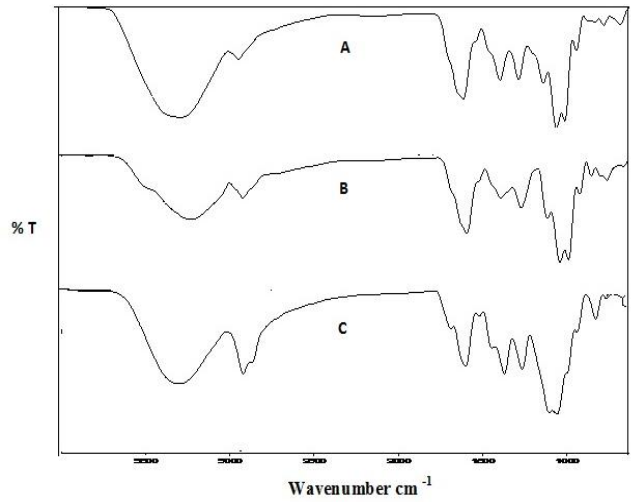

Figure 2: FT-IR spectroscopy of Coffee bean extract

The compatibility data of Coffee bean extract(Chlorogenic Acid) with potential formulation excipients studied by FT-IR spectroscopy is presented in Figure 2. The characteristics peaks of Coffee bean extract(Chlorogenic Acid) are retained. The peaks were observed at , (3048 cm^{-1})- Aromatic C-H Stretch, (3300 cm^{-1})- O-H, (2950 cm^{-1})- Aliphatic C-H Stretch, with only minor shifts in wave number thus suggesting that there is no interaction between the Coffee bean extract(Chlorogenic Acid) and other excipients. Hence drug-excipients compatibility was established.

3.1.5 Optimization and formulation of Coffee bean extract SLN:

Table 6: Experimental run and responses for optimization of Coffee bean extract SLN formula using Box- Behnken design.

Formulation Code	Run	Factor 1 (A): Concentration of Surfactant (Tween 80) (%)	Factor 2 (B): Homogenization pressure (bar)	Factor 3 (C): homogenization cycles (no.)	Response1 *Particle Size (nm)	Response 2 *Entrapment Efficiency (%)
5	1	2	800	3	545	86.29
8	2	6	800	9	430	88.25
3	3	2	1200	6	309	89.68
4	4	6	1200	6	210	91.18
1	5	2	400	6	660	75.47
10	6	4	1200	3	261	90.88
13	7	4	800	6	502	74.11
2	8	6	400	6	531	76.66
9	9	4	400	3	698	74.88
6	10	6	800	3	470	91.48
7	11	2	800	9	510	92.62
11	12	4	400	9	564	85.19
12	13	4	1200	9	249	90.2

***n=3 i.e. average of three readings**

3.1.5.1 Preparation of Coffee bean extract Solid Lipid Nanoparticles (SLN):

Coffee bean extract SLN were prepared by melt emulsification method followed by high pressure homogenization. Coffee bean extract(Chlorogenic Acid) SLN were prepared as per the experimental design given in the table 4 and were evaluated for particle size and entrapment efficiency. The Box-Behnken design reduces number of experiments in a 3- Factor, 3-Levels experimental design from 27 to 13. Another advantage of the Box-Behnken design is that it does not contain combinations for which all factors are simultaneously screened at their highest or lowest levels.

3.1.6 Characterisation of Coffee bean extract SLN

3.1.6.1 Determination of Particle size:

The particle size of SLN ranged between 698- 210 nm. It was seen that the concentration of tween 80 and homogenization pressure and number of homogenization cycles contributed in reduction of particle size. However with increase in homogenization cycles the particle size increased marginally this can be explained to high amount of collisions or very high surface area generated which can not be stabilized by available surfactant. On the other hand with decrease in particle size the migration of drug from emulsion globules to dispersion medium increased also aided by high temperature and surfactant concentration, resulting in reduction of drug entrapment.

The statistical model generated for the particle size

Particle Size = +502.00-47.88A-178.00B-27.63C-1.2546.90+7.50AB-1.25AC+30.50BC-$14.37A^2$+$60.12B^2$+$1.12C^2$

Equation shows that tween 80 had a limited effect of reducing the particle size, in comparison to homogenization pressure that can be attributed to high density fluid-dynamic energy conditions. The interaction term AB i.e. as the surfactant concentration and homogenization pressure contributed to decrease in particle size. Also at low concentration of tween 80 protective layer around the particle was not formed thus leading to the aggregation of particles.

The interaction term AC showed increase in particle size which is due to high surface area generated by more homogenization cycles which cannot be protected by surfactant and hence marginally the particle size is increased.

The interaction term BC i.e. high homogenization pressure and homogenization cycles the particle size was found to be decreasing. Similar effects were shown in figure 3, 4 and 5.

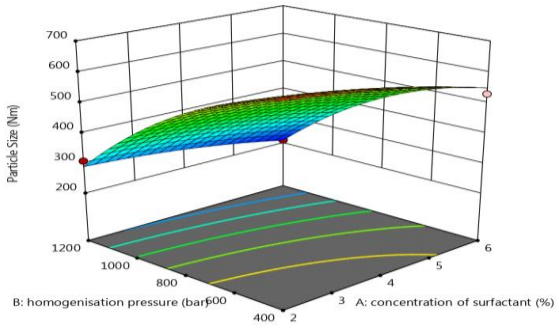

Figure 3: Response surface plot showing influence of Concentration of Surfactant and homogenization pressure on Particle size.

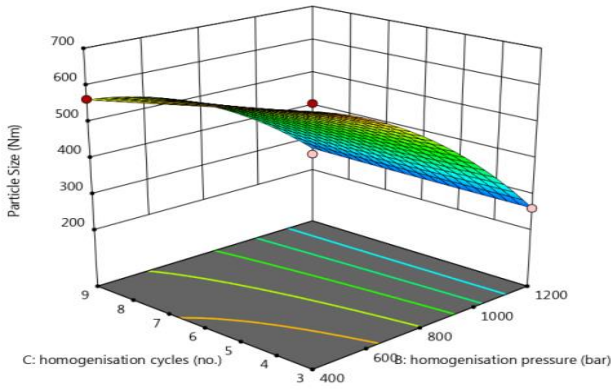

Figure 4: Response surface plot showing influence of homogenization pressure and homogenization cycle on Particle size.

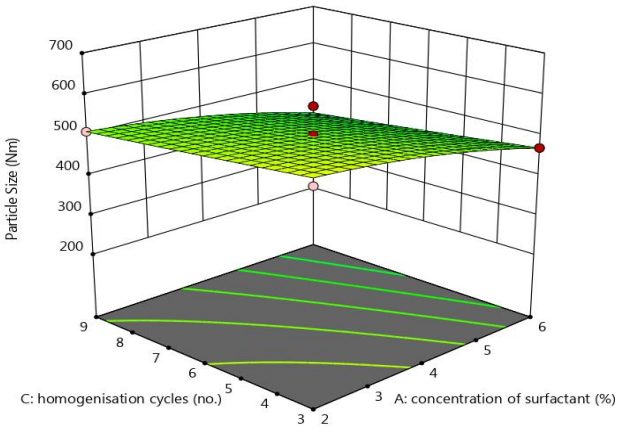

Figure 5: Response surface plot showing influence of Concentration of Surfactant and homogenization cycles on Particle size.

3.1.6.2 Determination of Entrapment Efficiency:

Equation 4 for Entrapment Efficiency:

$$\text{Entrapment Efficiency} = +74.11 + 0.4387A + 6.22B + 1.59C - 2.39AC - 2.75BC + 6.76A^2 + 2.38B^2 + 8.79C$$

Equation 4

The model indicates that as the concentration of surfactant goes on decreasing the drug incorporation ability increases and thus the entrapment efficiency increases Equation (4). As the particle size goes on decreasing the chances of drug expulsion increases and thus the entrapment efficiency decrease. On the other hand with decrease in particle size the migration of drug from emulsion globules to dispersion medium increased also aided by high temperature and surfactant concentration, resulting in reduction of drug entrapment.

The **interaction term AC** i.e. the concentration of surfactant and homogenization cycle was seen to contribute reduction in drug entrapment which is because lower particle size of globules leading to enhanced area of migration of drug to aqueous phase.

The **interaction term BC** i.e. Entrapment efficiency was not highly affected by homogenization pressure and homogenization cycle. Similar effects were shown in figure 6,7 and 8.

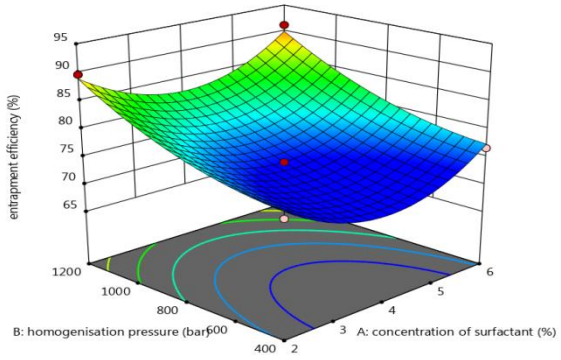

Figure 6: Response surface plot showing influence of Concentration of Surfactant and of homogenization pressure on Entrapment Efficiency.

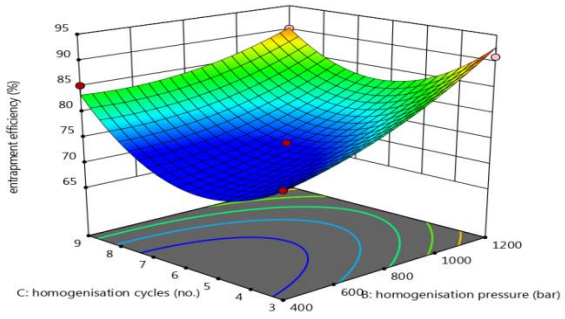

Figure 7: Response surface plot showing influence of Homogenization pressure and homogenization cycle on Entrapment Efficiency.

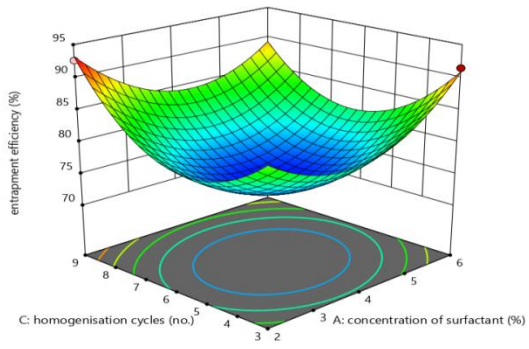

Figure 8: Response surface plot showing influence of Concentration of surfactant and homogenization cycle on Entrapment Efficiency.

3.1.6.3 Validation of the Response Surface Methodology (RSM):

To evaluate the findings of the RSM, verification run was carried out and no significant difference was found between the theoretical and the actual values of particle size and entrapment efficiency. The responses were evaluated for particle size and entrapment efficiency as shown in the table 7. The composition, actual and predicted values of the responses and the % prediction error are shown in the table below. Thus, the formulation batch giving minimum particle size and maximum entrapment was chosen as the optimized batch. Thus, the optimized value of surfactant concentration **5.9**%, homogenization pressure1200 bar and 3 homogenization cycles were predicted to yield SLN with least possible particle size and higher entrapment.

Table 7: Check Points for optimization, actual, predicted value and % error (n=3, Mean± SD)

Formulation Code	Composition of optimized formulation			Response	Predicted Value	Actual Value	% Error
	X1	X2	X3				
OF1	6	1200	9	Y1	209.0nm	210nm	1.0
				Y2	93.55%	91.18%	2.37

3.1.7 Evaluation of Optimized Solid Lipid Nanoparticles:

3.2.7.1 Determination of Particle Size and Entrapment Efficiency:

The particle size of Coffee bean extract SLN was seen to be increased from 178 nm of Blank SLNs to 210 nm and entrapment efficiency was 91.18 as seen in Table 8.

Table 8: The particle size, entrapment efficiency of Blank-SLN, Coffee bean extract-SLN are given in table. (n=3, Mean± SD)

Formulation	Particle Size (nm)	Polydispersity Index	Entrapment Efficiency
Blank SLNs	178	0.227	---
CGA-SLNs	210	0.455	91.18

3.1.7.2 Differential Scanning Calorimetry (DSC):

The melting endotherm of Chlorogenic acid and GMS was observed at 203ºC and 70.08ºC respectively (Figure 8). The DSC thermograms of Chlorogenic acid SLN showed an endotherm at 69 ºC this slight decrease in melting endotherm can be attributed to decreased crystallinity of GMS in SLN. However the disappearance of chlorogenic acid melting endotherm was proof of drug getting molecularly entrapped in the GMS matrix.

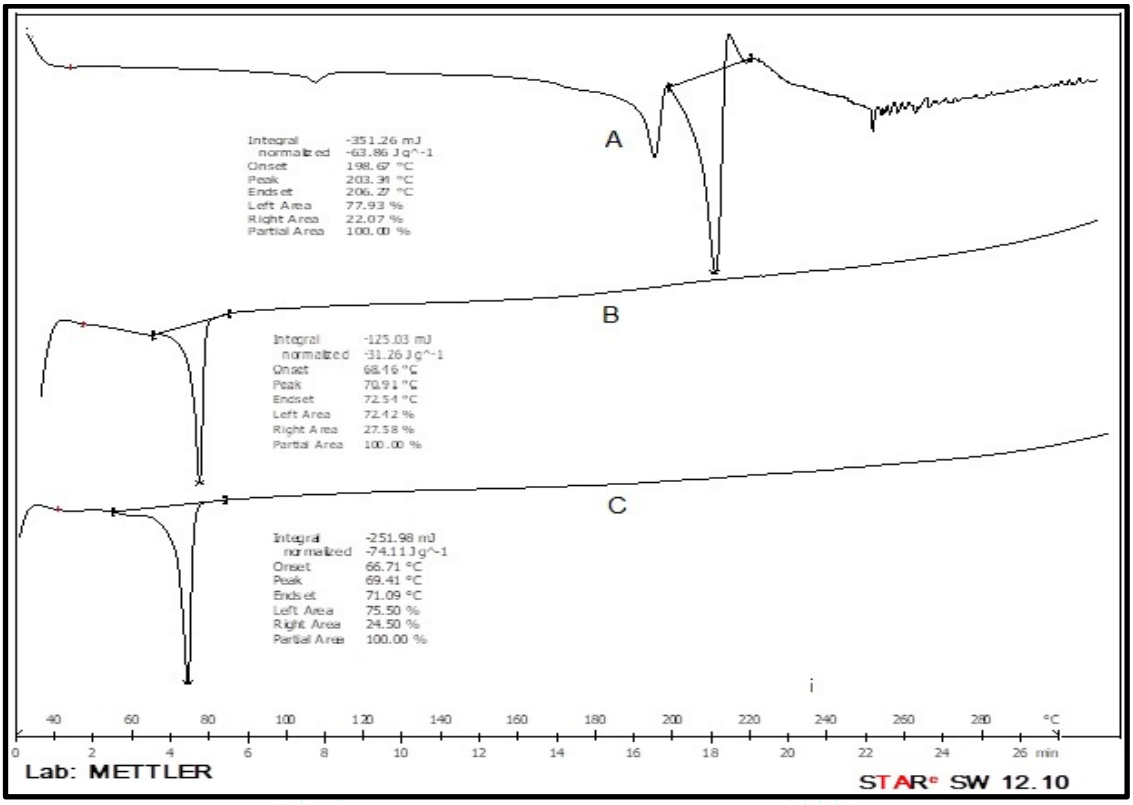

Figure 8: DSC thermograms of A] Chlorogenic acid , B] GMS and C] Chlorogenic acid Solid Lipid Nanoparticles (CGA-SLNs)

3.2 Ex-vivo Studies:

Numerous Literature[23,24]identifies energy dependent endocytic processes for the uptake of nanoparticles of which primary are clathrin-mediated endocytosis (receptor-mediated process), caveolae-mediated endocytosis (receptor-mediated process) and clathrin-caveolae independent process, importantly, micropinocytosis (actin dependent non receptor- mediated process). In micropinocytosis, large (0.5-2µm) vesicular structures at the cell surface called macropinosomes are formed; particle smaller than 2µm can be internalized into enterocytes by this mechanism[23]micropinocytosis being non-specific pathway for endocytosis uptake, it was not evaluated for uptake of Coffee bean extract(Chlorogenic Acid)-SLNs.[24]

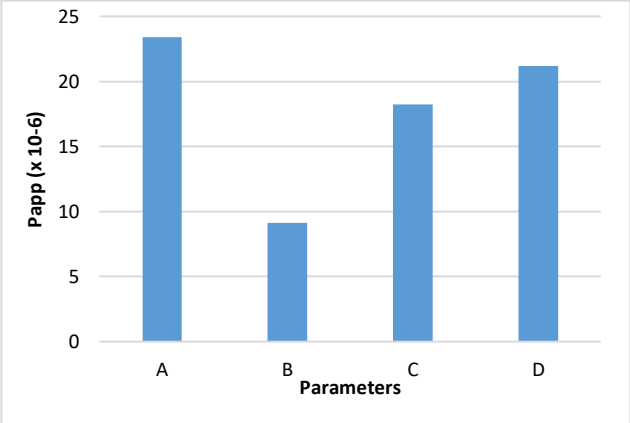

Figure 9: Ex-vivo apparent permeability of Coffee bean extract SLN at A) Coffee bean extract SLN at 37 ◦ C B) Coffee bean extract SLN at 4 ◦ C C) In presence of Chlorpromazine at 37 ◦ C, D) In Presence of Nystatin at 37 ◦ C

To explicate the cellular uptake pathways for Solid Lipid Nanoparticles, the experiment was carried out in the presence of inhibitors of clathrin-mediated endocytosis and caveolae-mediated endocytosis. Chlorpromazine, a cationic, amphiphilic drug, is reported to inhibit clathrin-mediated endocytosis by causing clathrin to accumulate in late endosomes and reducing its availability on the enterocytes surface[23]while caveolae, special type of lipid rafts rich in cholesterol and sphingo lipids are flask-shaped invagination on the plasma membrane to engulf cargo molecules or carriers binding to their surface. Antifungal drug nystatin causes cholesterol sequestration thus blocking the caveolae mediated uptake.[15]

Figure 9 represents the apparent permeability of the Coffee bean extractSLN at 37 ◦ C, at 4 ◦ C, in presence of Chlorpromazine at 37 ◦ C and in presence of Nystatin at 37 ◦ C measured for 30 minutes. It was evident from the results that the apparent permeability in presence of Chlorpromazine was reduced by 29.8% as compared to that in absence of Chlorpromazine. Similarly, the apparent permeability in presence of Nystatin was also found to be reduced 19.6% thus concluding the contribution of both clathrin and caveolae mediated process in the uptake of SLN. After application of single way ANOVA followed by Tukey's multiple comparison test, the differencebetween A, B, C, D and E was found significant ($p < 0.001$). The reduction in the apparent permeability at 4 °C by 76.2% in comparison with that at 37 °C also confirms the mechanism.

4. CONCLUSION:

The study investigated formulation optimization of SLN containing Coffee bean extract (Chlorogenic Acid) DMARD of phytochemical origin for lymphatic uptake which can reduce side effects and improve concentration of active at inflamed site in treatment of RA. The study through *ex vivo* study using everted rat gut sac model established that

Coffee bean extract (Chlorogenic Acid) SLN with particle size **210** nm of Coffee bean extract SLN can effectively be taken up by lymphatic route, as demonstrated by lack of uptake in presence of lymphatic uptake blockers.

Declaration of interest:

'Declarations of interest: none'.

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