6. RESULTS

a. Pre-formulation studies

(I). Analytical method development by UFLC for DADS

Calibration curve of DADS exhibited perfect linearity between the concentration of drug and absorbance when observed at the concentration range between 1mM-10mM (Table 4) and (Figure. 2). The linear regression equations for DADS was $y = 13182x - 49208$ with correlation coefficient ($r^2$) values of 0.999.

**Table 4. Concentrations and average peak area obtained for the development of calibration curve of DADS**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Concentration (mM)</th>
<th>Average Peak area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>1</td>
<td>926355.5</td>
</tr>
<tr>
<td>2.</td>
<td>2</td>
<td>2048675</td>
</tr>
<tr>
<td>3.</td>
<td>4</td>
<td>4841703</td>
</tr>
<tr>
<td>4.</td>
<td>6</td>
<td>7310328</td>
</tr>
<tr>
<td>5.</td>
<td>8</td>
<td>10015155</td>
</tr>
<tr>
<td>6.</td>
<td>10</td>
<td>12769150</td>
</tr>
</tbody>
</table>

(A)
Figure 2. (A) Analytical Calibration curve development of DADS (B) chromatogram of DADS

(II). Partition coefficient study
DADS is lipophilic drug (Log P-value = 2.55) (85). Reason for selecting monoglycerides such as Palmitic acid and Stearic acid as due to the presence of free carboxyl groups facilitates the folic acid conjugation by carboxyl-amine conjugation. Partition coefficient of DADS was high in Palmitic acid than Stearic acid (Table 5). So, Palmitic acid-monoglyceride fatty acid was selected for the development of SLN to attain the optimum sustained release of DADS.

Table 5. Partition coefficient of DADS in different solid lipids

<table>
<thead>
<tr>
<th>Lipids</th>
<th>Drug in water phase (mg)</th>
<th>Drug in lipid phase (mg)</th>
<th>Partition coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stearic acid</td>
<td>4.12 ± 0.09</td>
<td>5.88 ± 0.07</td>
<td>1.42</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>4.08 ± 0.035</td>
<td>5.92 ± 0.06</td>
<td>1.45</td>
</tr>
</tbody>
</table>
Results

(III). Drug - lipid interaction study by Fourier Transform Infra-Red (FTIR)
Spectral Analysis

Compatibility study was carried out by FT-IR spectroscopy. IR spectrum of naïve DADS exhibited the absorption bands at 3082.35 cm\(^{-1}\) (=C-H stretch), 2979.16 cm\(^{-1}\) (C–H stretch), 721.40 cm\(^{-1}\) (C–H rock) (Figure 3). These are the signature peaks of DADS. For Palmitic acid, absorption bands were observed at 2917.43 cm\(^{-1}\) (C–H stretch), 2660.89 cm\(^{-1}\) (O–H stretch) 1692.59 cm\(^{-1}\) (C=O stretch) and 933.58 cm\(^{-1}\) (O–H stretch). Absorption peaks of DADS SLN were appeared at 3082.35 cm\(^{-1}\), 2955.04 cm\(^{-1}\), 2656.07 cm\(^{-1}\), 1705.13 cm\(^{-1}\), 918.15 cm\(^{-1}\) and 720.44 cm\(^{-1}\) respectively indicating vibrations of =C-H stretch, C–H stretch, O–H stretch, C=O stretch , O–H stretch and C–H rock haven’t altered. The IR spectrum of drug–lipid physical mixture showed that palmitic acid did not affect DADS signature bands. Compatibility studies were carried out for the DADS-SLNs showed no disappearance of the peaks or peak shifts, indicating that the drug is compatible with ingredients in the SLN formulation.

![Figure 3. FT-IR spectra of (A) DADS (B) Palmitic acid (C) Physical mixture (D) DADS-SLNs](image)

b. Preparation of DADS-SLNs
(I). Development of statistically optimized DADS-SLN by Response surface methodology – Box behnken design

The responses of the experimental design were statistically analyzed using Design-Expert® (Version 7.1.6, Inc, USA Stat-Ease Inc., MN,). It provided ample-sized valuable data for selection of optimization design and analysis of variables.
Independent variables selected for the optimization were concentration of surfactant, volume of solvent and amount of lipid as they played major role in development of DADS-SLNs. These formulation variables controlled the dependent variables particle size (PS) and entrapment efficiency (EE). Polynomial equations generated by the software indicate main and interaction effects of independent variables. These effects are developed on the basis of the predicted residual sum of squares, multiple correlation coefficients and adjusted multiple correlation coefficients. Polynomial equations are statistically validated by ANOVA dialogue box present in analysis provision of the software. Thereby, values of the independent variables and dependent variables of optimized formulation were generated by experimental data inserted in the Design-Expert software (Table 6). Perturbation plots were constructed to find the main effect of the independent variables influencing dependent variables (Figure. 4) the model polynomial functions, to determine change in the response surface. These plots infer function of each variable on each response which can be perceived by the developed celluloid.

i. Analysis of Responses
The responses obtained from 17 formulations were fitted into the experimental design present in the Design-Expert software.

ii. Response Y₁ (Particle size)
The ratio of higher value to lower value of response Y₁ was 1.68 which doesn’t required power transformation. Transformation of response plays vital role in data interpretation. The thumb rule of power transformation in responses is necessary and ratio of higher value to lower value should be less than 10 and greater than 3. To analyze the responses the model was selected on the basis of lack of fit, sequential model sum of squares and model summary statistics. For Y₁ response, quadratic model was suggested as per the P < 0.0001, lower predicted residual error sum of square (PRESS), high R-squared and low standard deviation. ANOVA confirmed quadratic model of Y₁ response was significant Model Prob > F < 0.05. The F value of response Y₁ reported to be 818.66, inferring the quadratic model was significant. ANOVA recognizes the concentration of surfactant, amount of lipid and volume of solvent as significant model terms that affect the Y₁ response (P<0.05). Lack of fit F-value for Y₁ was 0.96 which infers that it was not significant and it is relative to the pure error. Response Y₁ exhibited the predicted R-squared value of 0.9928 and adjusted R-squared value of 0.9978
Table 6. Responses obtained for selected parameters from experimental batches.

<table>
<thead>
<tr>
<th>Run</th>
<th>Factor 1</th>
<th>Factor 2</th>
<th>Factor 3</th>
<th>Response 1</th>
<th>Response 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration of surfactant (%)</td>
<td>Amount of lipid (mg)</td>
<td>Volume of organic solvent (ml)</td>
<td>Particle size (nm)</td>
<td>Entrapment efficiency (%)</td>
</tr>
<tr>
<td>1</td>
<td>1.00</td>
<td>75</td>
<td>2.00</td>
<td>129</td>
<td>55.85</td>
</tr>
<tr>
<td>2</td>
<td>1.50</td>
<td>50</td>
<td>4.00</td>
<td>112.1</td>
<td>56.75</td>
</tr>
<tr>
<td>3</td>
<td>1.50</td>
<td>75</td>
<td>3.00</td>
<td>135.2</td>
<td>65.17</td>
</tr>
<tr>
<td>4</td>
<td>2.00</td>
<td>75</td>
<td>4.00</td>
<td>121.8</td>
<td>74.68</td>
</tr>
<tr>
<td>5</td>
<td>1.50</td>
<td>100</td>
<td>4.00</td>
<td>131.35</td>
<td>73.28</td>
</tr>
<tr>
<td>6</td>
<td>1.50</td>
<td>100</td>
<td>2.00</td>
<td>171.2</td>
<td>80.77</td>
</tr>
<tr>
<td>7</td>
<td>2.00</td>
<td>50</td>
<td>3.00</td>
<td>102.8</td>
<td>54.33</td>
</tr>
<tr>
<td>8</td>
<td>1.00</td>
<td>75</td>
<td>4.00</td>
<td>114.7</td>
<td>48.78</td>
</tr>
<tr>
<td>9</td>
<td>1.50</td>
<td>75</td>
<td>3.00</td>
<td>134.2</td>
<td>65.9</td>
</tr>
<tr>
<td>10</td>
<td>1.50</td>
<td>75</td>
<td>3.00</td>
<td>133.9</td>
<td>65.5</td>
</tr>
<tr>
<td>11</td>
<td>1.50</td>
<td>75</td>
<td>3.00</td>
<td>134.7</td>
<td>63.11</td>
</tr>
<tr>
<td>12</td>
<td>1.00</td>
<td>100</td>
<td>3.00</td>
<td>135.4</td>
<td>70.02</td>
</tr>
<tr>
<td>13</td>
<td>1.00</td>
<td>50</td>
<td>3.00</td>
<td>101.6</td>
<td>34.8</td>
</tr>
<tr>
<td>14</td>
<td>1.50</td>
<td>50</td>
<td>2.00</td>
<td>109.2</td>
<td>39.44</td>
</tr>
<tr>
<td>15</td>
<td>1.50</td>
<td>75</td>
<td>3.00</td>
<td>133</td>
<td>65.54</td>
</tr>
<tr>
<td>16</td>
<td>2.00</td>
<td>100</td>
<td>3.00</td>
<td>152.4</td>
<td>75.99</td>
</tr>
<tr>
<td>17</td>
<td>2.00</td>
<td>75</td>
<td>2.00</td>
<td>141.3</td>
<td>59</td>
</tr>
</tbody>
</table>

Predicted R-squared and adjusted R-squared values were in close agreement inferring the model is fit. Adequate precision for response Y1 was 109.54. Adequate precision determines the signal to noise ratio. The thumb rule for adequate precision is that ratio should be greater than 4 is desirable. Quadratic model obtained for the response Y1 facilitates navigation in the design space. The quadratic equation generated as per the coded factors for response Y1 is denoted in equation. (1)

\[
Y_1 = 134.20 + 4.70X_1 + 20.58X_2 - 8.84X_3 + 3.95X_1X_2 - 1.30X_1X_3 - 10.69X_2X_3 - 7.71X_1^2 - 3.44X_2^2 + 0.21X_3^2
\]

This regression equation expresses the quantitative terms of the independent variables (X1, X2, X3). X1, X2, X3 represent main effects influencing the response Y1. X1X2, X1X3, X2X3, X1^2, X2^2 and X3^2 represent interaction effects and variables with second-order factors describe nonlinear connection between the dependent variable and independent variable. Positive sign and negative sign indicate the synergistic and antagonistic effects respectively on the response Y1. Response Y1 was favorable with variables X1, X2 and detrimental effect with variable X3.
Perturbation graphs were constructed for finding the each variable’s solo influence on the response (Figure 4). Curvaceous characteristic line implies that response is flexible for optimization whereas variable with rectilinear line implies the response is not favourable for optimization. If two or more variables exist, then the perturbation graph is capable in determining the variable has most affect over the response. For response \( Y_1 \), factor A shows noticeable slope; factor B shows steep slope and factor C is lesser slope. This infers that surfactant and lipid have influence on the particle size. For response \( Y_2 \), factor A and factor B show steep curvature, whereas factor C shows less curvature. Similarly, lipid and surfactant determines the entrapment efficiency. It is evident that concentration of surfactant increased the particle size significantly. In the emulsification phenomenon, high sheared droplet size tends to minimize and undergoes aggregation to diminish their surface energy. However, presence of surfactant brings stability to the emulsion by enveloping the droplet to avoid the aggregation. When surfactant concentration increased, particle size increase was observed from lower to mid level (-1 to 0) and later it got declined. After certain extent, surfactant concentration exhibited inverse relation on the particle size. The reason might be decreased surface tension between aqueous phase and lipid phase which formed space between them and higher surface area formation. The strong positive coefficient of \( X_2 \) indicates particle size is directly proportional to lipid concentration. This has a close concord with the postulate of Muller–Goymann (86).

Mathematical interconnection between factors and responses is illustrated by response surface plots. Interaction effects of variable \( X_1 \) and variable \( X_2 \) was analyzed by making variable \( X_3 \) at constant level, interaction effects of variable \( X_1 \) and variable \( X_3 \) and their effect was analyzed by keeping variable \( X_2 \) at constant level and the interaction effect of variable \( X_2 \) and variable \( X_3 \) were studied when variable \( X_1 \) was made constant on response \( Y_1 \) were shown in (Fig. 5 A, B, C), respectively.

Simultaneous elevation of lipid and surfactant concentrations exhibited positive impact (coefficient of \( X_1X_2 = + 3.95 \)) on particle size which was illustrated in (Figure 5(A)) while simultaneous increase of surfactant and solvent also shown positive impact on particle size (coefficient of \( X1X_3 = – 1.30 \)) (Figure 5(B)). When lipid and solvent were simultaneously increased, an irrelevant effect was observed on particle size (coefficient of \( X_2X_3 = – 10.69 \)) shown in (Figure 5C) where \( X_2 \) alone showed positive relationship but \( X_3 \) alone has inverse effect on particle size. Particle size is the deciding factor which has influence on entrapment, loading, release, efficacy and bioavailability. The most challenging issue in designing nanoparticles is achievement
Results

of narrow size distribution which depends on narrow droplet size distribution. SLNs undergo cellular internalization by endocytosis; here particle size has inverse relationship on cellular uptake and influence the drug bioavailability.

iii. Response $Y_2$ (Entrapment efficiency)

Power transformation is not required for the $Y_2$ response as the ratio of higher limit to lower limit was 2.32. Lack of fit model, sequential model sum of squares and model summary statistics have suggested quadratic model to the response $Y_2$ for the analysis. The Prob $> F$ value of $P < 0.0001$, high $R^2$-squared, lower PRESS values and low standard deviation. For the response $Y_2$, ANOVA of the model was significant (Model Prob $> F$ less than 0.05). For response $Y_2$, the Model $F$ value was 285.06, which defines the model was significant. All the variables were significant and influence the $Y_2$ response ($P<0.05$).

Lack of fit $F$-value for $Y_2$ response was 0.51 inferring that it has no significant relative to the pure error. For the response $Y_2$, predicted $R^2$-squared and adjusted $R^2$-squared values were 0.9849 and 0.9938 respectively. This indicates that predicted $R^2$-squared value and adjusted $R^2$-squared value are in good agreement indicating a good fit. Adequate precision for response $Y_2$ was 61.503 indicates that it is an adequate signal.

The quadratic equation obtained as per coded factors is presented in equation. (2)

$$Y_2 = 65.04 + 6.82 X_1 + 14.34 X_2 + 2.30 X_3 - 3.39 X_1 X_2 + 5.69 X_1 X_3 + 6.20 X_2 X_3 - 4.62 X_1^2 - 1.64 X_2^2 - 0.85 X_3^2$$
Results

Figure 4. Perturbation plots of response $Y_1$ particle size and response $Y_2$ entrapment efficiency.

All the variables were favorable to the response $Y_2$ which was observed in the regression equation. Among the three variables, $X_2$ has high positive regression coefficient implying that lipid proportion has crucial role in governing drug entrapment. This might be attributed due to long chain fatty acids of lipid. DADS being a oily drug can develop deformities in the lipid matrix and enhance the entrapment. DADS has a pronounced hydrophobic character that may account for the higher association with the lipid and further enhance the drug encapsulation in the lipid matrix.
Figure 5. 3-D surface plots (A) amount of lipid and concentration of surfactant on $Y_1$, (B) volume of organic solvent and concentration of surfactant on $Y_1$ and (C) volume of organic solvent and concentration of lipid on $Y_1$.

The mathematical interconnection between the factors and response $Y_2$ was expressed using the 3-D surface plots as shown in (Figure. 6 A, B, C). Even the 3-D surface plots have illustrated that all variables were significant and showed positive impact on entrapment efficiency. The negative regression coefficient for simultaneous increase of $X_1$ and $X_2$ indicated that the surfactant concentration and the amount of lipid lowered the drug entrapment efficiency. Pluronic® F-68 is a
surface modifying agent which can control porosity of lipid component. So, this may render exclusion of entrapped drug to the secondary aqueous phase and led to low entrapment efficiency. Increasing $X_2$ and $X_3$ simultaneously significantly influenced the entrapment efficiency. And even the same positive response observed when there is simultaneous increase of $X_2$ and $X_3$.

iv. Optimization and Validation
The desirability criterion was explored by the Design-Expert software to achieve the desirable optimized formulation. Optimized DADS-SLNs was developed by fixing the set criteria of minimal particle size and maximal entrapment efficiency. Using the predicted values of lipid, surfactant and solvent compositions, a batch of DADS-SLNs was formulated to validate optimization protocol. The proportions of optimized DADS-SLNs were 2.23% (w/v) surfactant concentration, 64.15 mg of lipid and 4 ml of solvent comply the requirements of optimized DADS-SLN (Figure. 7). The optimized DADS-SLNs showed $108.112 \pm 0.57$ nm (Figure. 8A) and entrapment efficiency $71.806 \pm 0.14$ % which were in close concord with the predicted values. Zeta potential and drug loading of DADS-SLNs were $-7.7$mv and $34.72333 \pm 1.000417$. SEM analysis reported that DADS-SLNs were utmost uniform-sized; mono-dispersed spherical shaped. It was observed majority of SLNs showed slight rough surface morphology. (Figure. 8C)
Figure 6. 3-D surface plots (A) amount of lipid and concentration of surfactant on Y₂, (B) volume of organic solvent and concentration of surfactant on Y₂ and (C) volume of organic solvent and concentration of lipid on Y₂.
c. Physicochemical characterization of DADS-SLN and FA-DADS-SLN

(i). Particle size, Zeta Potential, Entrapment efficiency and Drug loading

Dynamic light scattering reports suggests that FA-DADS-SLN and DADS-SLN were in average size range of 167± 1.72 nm and 108.112 ± 0.57 nm which were in acceptable limits (Figure. 8A). Compared with DADS-SLN, the particle size was higher in FA-DADS-SLN which might be attributed due to the surface functionalization (Figure. 8A & 8B). Polydispersity index (Pdi) of FA-DADS-SLN and DADS-SLN were 0.119 ± 0.010817 and 0.124 ± 0.005568 (Pdi<0.2) which indicates the good redispersibility of developed nanoparticles. Zeta potential of FA-DADS-SLN and DADS-SLN was 3.6mv and -7.7mv respectively. The underlying reason in the zeta potential shift may be due to the negatively charged carboxyl group of Palmitic acid conjugation with folic acid by amide bond formation. This phenomenal suggests the proper conjugation between amine group of folic acid and carboxyl group of Palmitic acid.SEM studies reported that FA-DADS-SLN was monodispersed and spherical shaped (Figure. 8D). Slight rough surface morphology was observed. The entrapment efficiency of FA-DADS-SLN and DADS-SLN was found to be 69.76 ± 0.11% and 71.96 ± 0.55 % respectively. The drug content of FA-DADS-SLN and DADS-SLN was found to be 34.72333 ± 0.115 % and 29.75± 0.44658 %. No significant difference was observed in the entrapment efficiency between FA-DADS-SLN and DADS-SLN.
Results

Figure 8. (A) Particle size distribution analysis of DADS-SLNs (B) Particle size distribution analysis of FA-DADS-SLNs (C) SEM image of DADS-SLNs (D) SEM image of FA-DADS-SLNs.

(II). Characterization of Folic acid conjugation to DADS-SLNs by FT-IR analysis

Conjugation of the Palmitic acid and Folic acid was confirmed by the FTIR analysis

Figure 9. FT-IR spectra of (A) Palmitic acid (B) Folic acid (C) FA-DADS-SLNs
Results

(Figure.9). Folic acid exhibited bands at 3320.94 cm\(^{-1}\) and 3408.54 cm\(^{-1}\) which indicates the presence of the –NH\(_2\) groups. For Palmitic acid, IR absorption peaks were recorded at 2917.43 cm\(^{-1}\) (C–H stretch), 2660.89 cm\(^{-1}\) (O–H stretch) 1692.59 cm\(^{-1}\) (C=O stretch) and 933.58 cm\(^{-1}\) (O–H stretch). The FTIR spectra of FA-DADS-SLNs showed a stronger band at 3531.47 cm\(^{-1}\) which indicates the presence of aromatic secondary amine group that indicates the successful conjugation by the (–NH\(_2\)) groups of folic acid with (–COOH) groups of palmitic acid.

(III). In vitro drug release study

There was no burst release observed up to 24 h and exhibited sustained release pattern in all groups (Figure.10). FA-DADS-SLNs released drug significantly slower than DADS-SLNs because of the enveloping of folate moiety on the lipid surface. Lipids enhance the rigidity of the encapsulation layers. So, surface conjugation of the lipid altered the flexibility of the lipid layer and delayed the rate of drug release. Aberrant glycolysis, elevated lactic acid generation and interrupted convective transport, sync together stack up H\(^+\) ions in cancer cells. Pronouncedly, in large and
Results

/or small-flow tumors pH shift occurs towards the acidic range (87). Extracellular acidosis and elevated p-glycoprotein activity turns the tumors into chemo-resistant phenotype (88). To mimic the pH variation of normal/healthy cell exterior (pH 7.4) and tumor cellular lysosome (pH 4.5), drug release study was carried out at different pH environments. As clearly described in (Fig. 9), higher amount and burst release of DADS release was observed at lower pH. DADS is a weak acid and alkaline in nature due to the pair of sulfide groups and soluble at acidic pH. Thereby, encapsulated DADS inside SLNs tend to relocate into acidic medium.

d. In vitro cell line studies

(i). In vitro cytotoxicity studies

The in vitro cytotoxic activity of DADS, DADS-SLNs and FA-DA-SLNs were evaluated by the SRB assay, the cell viability are shown in (Figure.11). Blank FA-SLNs exhibited no toxicity to MCF-7 cells which confirms the safety of the nanoparticles. MCF-7 cells treated with DADS-SLNs and FA-DA-SLNs exhibited cytotoxicity at various concentrations (1.562, 3.125, 6.25, 12.5, 25, 50, 100 µM). It is evident that DADS, DADS-SLNs and FA-DA-SLNs exhibited dose-dependent cytotoxic action. DADS-SLNs had exhibited lower cytotoxic action when compared

![Graph showing in vitro cell viability of MCF-7](image)

Figure 11. In vitro cytotoxicity study by SRB assay (A) Dose dependent cytotoxicity of DADS, DADS-SLNs and FA-DADS-SLNs against MCF-7 cell lines. Data was presented as mean ±S.D. (n = 3). (*) p < 0.05, FA-DADS-SLN versus DADS.
(B) Dose dependent cytotoxicity of DADS, DADS-SLNs and FA-DADS-SLNs against MCF-10A cells. Data is presented as mean ± S.D. (n = 3).

with FA-DA-SLNs which might be due to the P-glycoprotein (P-gp) pumps effluxing of the diffused drug. FA-DA-SLNs might be internalized via folate receptor endocytosis and have no link with P-gp efflux. This leads to sustain presence of drug inside cells which exhibit high cytotoxic action (89,90). DADS, DADS-SLNs, FA-DADS-SLNs exhibited negligible cytotoxicity in MCF-10A cell lines (Figure. 11) and even blank SLNs has no cytotoxic effect in MCF-7 cells which confirms the safety of the nanoparticles.

(II) Measurement of reactive oxygen species
The intracellular ROS was determined with different concentrations of 1.562, 3.125, 6.25, 12.5, 50, 100 µM DADS, DADS-SLNs and FA-DA-SLNs. As shown in (Figure. 12) dose-dependent increase of ROS production was observed when MCF-7 cells treated with 1.562-100 µM DADS. When compared with DADS-SLNs, FA-DA-SLNs have shown slight enhancement in the fluorescent intensity.
Figure 12. ROS estimation study for FA-DADS-SLNs, DADS-SLNs and DADS. Data as mean ± S.D. (n = 3). (*) p < 0.05, DADS versus FA-DADS-SLNs.

(III) In vitro cellular uptake study by triple fluorescence staining method
Cellular uptake of the FA-DA-SLNs and DADS-SLNs was evaluated in MCF-7 breast

Figure 13. Confocal microscopic images of representing the cellular uptake of DADS-SLNs and FA-DADS-SLNs in MCF-7 cell lines.
cancer cells by triple fluorescence staining method. This method is carried out by 3 stains in which the red fluorescence emitted by the Nile Red which labels SLNs, green fluorescence emitted by ER-Tracker™ Green which labels the actin and endoplasmic reticulum, and blue fluorescence emitted by DAPI labels the nucleus. The lipophilic stain Nile Red is poorly water soluble stain (<1 μg/mL) labels the SLNs present inside the tumor cells. FA-DADS-SLN & DADS-SLN which were internalized in MCF-7 cells was illustrated in images Row 1 and 2 respectively (Figure. 13). Images in the Row 1 represented the passive or less targeting effect of DADS-SLN and images in the Row 2 represented the FA-DA-SLN targeting effect. Thereby, it was observed from the images that blue fluorescence indicating the nucleus stained by DAPI and red fluorescence representing the DADS-SLN/FA-DADS-SLN internalized in the cytoplasm. In the interim, the cytoskeleton and endoplasmic reticulum of the breast cancer cells were clearly visualized by green fluorescence stained by ER tracker. Thereby, the qualitative cellular uptake of DADS-SLN and FA-DADS-SLN were visually verified by the CLSM images.

(IV).Quantitative determination of apoptosis by annexin V/propidium iodide dual staining method
Annexin V dye labels the extrinsic phosphatidyl serine (PS) with fluorescence which differentiates apoptotic cells from live cells. Another fluorescent dye PI permeates into necrotic cells and could not internalize into live cells. So, using these two dyes the early and late apoptotic cells can be identified and projected into the four quadrants generated by the flow cytometer. Necrotic cells appear in first quadrant (Q1) as these cells accept PI alone, late apoptotic cells appear in second quadrant (Q2) as they get stained by PI as well as annexin V, early apoptotic cells appear in the third quadrant (Q3) as they accept annexin V alone and normal cancer cells (non-apoptotic & non-necrotic) appear in the fourth quadrant as they do not accept any of the stain. The stains differentiate the cell population of different stages. Apoptosis was quantitatively analyzed by the proportion of the gated events illustrated in (Figure. 14), early apoptotic and late apoptotic cells of control were 0.6% and 0.9%, while that of the FA-DADS-SLN were 5.7 % and 61.8%, which were higher than for both the DADS (1.49% and 14.72%) and the DADS-SLN (3.2 % and 54.2 %). Apoptosis enhancement was evident after the treatment at 5 μM concentration (24 h). FA-DADS-SLN exhibited significant apoptotic effect in comparison with naïve DADS and DADS-SLN. Therefore, we conclude that folate surface conjugation and nano encapsulation of DADS could enhance the apoptosis.
Figure 14. Quantitative measurement of apoptosis in MCF-7 cells treated with DADS, DADS-SLNs and FA-DADS-SLNs. (A) control (B) DADS (C) DADS-SLNs (D) FA-DADS-SLNs. Results of apoptosis were represented as plot of AnnexinV-FITC versus PI.

(V). Detection of apoptotic signaling pathways – Western blot analysis
Apoptosis blockade is one of the hallmarks in cancers. There are various tumor cell death mechanisms such as cell cycle arrest, anti-angiogenesis, anti-metastatic and autophagy but majority of the anti-cancer drugs exhibit cytotoxic effect apoptotic signaling pathways in tumors. Apoptotic signaling pathway is controlled by cascade reactions of complex molecules in the network which is associated with expression change of distinct pro-apoptotic and anti-apoptotic proteins. Bcl-2 family proteins comprises of pro-apoptotic proteins (Bim, Bax and Bad) and anti-apoptotic proteins (Bcl-xL, Mcl-1 and Bcl-2). These proteins involved in relocation the mitochondrial mediators and upregulation of caspases. Overexpression of the anti-apoptotic protein Bcl-2 blocks mitochondrial outer membrane permeabilization and inhibits apoptosis (91-93). Several investigations have well-documented that DADS induces apoptosis through the intrinsic apoptosis pathway (24) (65). To further understand
the molecular mechanism of the action of FA-DADS-SLNs in breast cancer cells, we assessed it's on the major apoptotic pathway. In our study, FA-DADS-SLNs exhibited elevation of Bax, Bad, caspase-9, caspase-3 levels and decreased expression of anti-apoptotic protein Bcl-2 when compared with DADS-SLNs and free-DADS (Figure.15). This suggests that FA-DADS-SLNs induced cell apoptosis through intrinsic signaling pathway.

Figure 15. Mitochondrial mediated apoptosis induced by FA-DADS-SLNs in comparison with DADS-SLNs and DADS treated with MCF-7 cells was confirmed by Western blot analysis of apoptotic related protein expressions.