7. DISCUSSION

Of the most prevailing diseases in the world, cancer can be noted. Among the cancers, breast cancer has severely affected in women and treatment & diagnosis made burdensome with its multi-factorial pathophysiology. With the available contemporary diagnostic tools and use of adjuvant systemic treatment, significant advancement has been taken place in improvement of overall survival rate at early stage breast cancer treatment (94). Current therapies for breast cancer are radiation therapy, chemotherapy and endocrine therapy which have exhibited significant therapeutic effect but toxicity and side effects are persisting which hinders the clinical utility (3). Most of the cytotoxic drugs used clinically are chemotherapeutics administered into systemic circulation. Chemotherapeutics are vulnerable for the development of drug-resistance, alopecia and cardio-toxicity(4).

Importance of nanotechnology to medical science paved several interdisciplinary fields among pharmacology, bio-chemistry, molecular biology, dentistry, toxicology and made path-breaking to solve the medical problems (14). Among the nanocarriers, lipid-based nanocarriers have numerous advantages which make it as an idealistic drug delivery system. They are bio-compatible, bio-degradable and can solubilize, encapsulate, protect & deliver active molecules in a programmed pattern to achieve higher therapeutic effect, bioavailability and avoid side-effects (35,36).

In this context, SLNs were prepared using bio compatible lipid Palmitic acid in different proportions. DADS exhibited high partition coefficient in Palmitic acid. Palmitic acid was subjected to characterization studies for cross compatibility along with DADS. The characterization studies include IR spectroscopy which had revealed no cross reactivity between DADS and Palmitic acid. DADS-SLNs were statistically optimized by utilizing the quality-by-design approach - Box behnken design. Folic acid conjugation was carried out by the carboxyl-amine reaction between carboxyl group of Palmitic acid and amine group of Folic acid. FA-DADS-SLNs and DADS-SLNs were in average size range of 167± 1.72 nm and 108.112 ± 0.57 nm. The particle size increase in FA-DADS-SLNs might be attributed due to the surface functionalization. Polydispersity index (Pdi) of FA-DADS-SLNs and DADS-SLNs were 0.119 ± 0.010817 and 0.124 ± 0.005568 (Pdi<0.2) indicating the redispersibility of developed nanoparticles. Shift of zeta potential of was observed in FA-DADS-SLNs 3.6mv and DADS-SLNs -7.7mv which might be due to the presence of amide bond formation. Slight rough surfaced, mono-dispersed and spherical
shaped confirms the structural aspects of FA-DADS-SLNs. The entrapment efficiency of FA-DADS-SLNs and DADS-SLNs was 69.76 ± 0.11% and 71.96 ± 0.55% respectively. The drug content of FA-DADS-SLNs and DADS-SLNs was found to be 34.72333 ± 0.115% and 29.75 ± 0.44658%. Significant difference was not observed in the entrapment efficiency and drug content between FA-DADS-SLNs and DADS-SLNs. Both developed formulations haven’t showed any burst release at 7.4 pH which indicates the controlled drug release from SLNs. But, burst release was observed at pH 4.5 in the acidic medium. Rapid drug release was attributed by the alkaline nature of the drug which was trying to enter into the acidic environment and later the slow release might be caused by diffusion of the drug from the lipid matrix of nanoparticles.

In vitro cytotoxic activity of DADS, DADS-SLNs and FA-DADS-SLNs were evaluated by the SRB assay. DADS encapsulated in the SLNs and FA-SLNs was examined for cytotoxicity against MCF-7 cell lines and MCF-10A cell lines. IC₅₀ values of FA-DADS-SLNs, DADS-SLNs and DADS were 4.991 µM, 8.843 µM and 12.46 µM. FA-DADS-SLNs induced significant cytotoxic action in MCF-7 cell lines which may be due to elevated intracellular uptake by the folic acid through folate receptor-mediated endocytosis. DADS-SLNs and FA-DADS-SLNs exhibited negligible cytotoxicity in MCF-10A cell lines and even blank SLNs has no cytotoxic effect in MCF-7 cells which confirms the safety of the nanoparticles. FA-DADS-SLNs internalized into MCF-7 cells via receptor mediated endocytosis. This leads to sustain presence of drug inside cells which exhibit high cytotoxic action (89,90).

FA-DADS-SLNs showed significant elevation of ROS generation because DADS exhibit apoptosis mediated cell death by increased levels of ROS (95). The prime-motivest aim in developing a delivery system for chemotherapeutic is tumor targeting while neglecting the healthy cells and this will ensure extensive drug uptake in cancerous tissue. Endocytosis mediated system is one of the important internalization pathway in tumors. Qualitative cellular uptake study had portrayed the internalization of FA-DADS-SLNs and DADS-SLNs (96). Thereby, internalization of FA-DADS-SLNs and DADS-SLNs was studied by triple fluorescence staining method. Its been clearly witnessed the nuclei was visualized by DAPI (blue dye), the endoplasmic reticulum and actin labeled by ER-Tracker™ Green (green dye) and Nile red labeled the SLNs. Green and red fluorescence overlapping gave yellow staining which was the co-localization of SLN with ER. The higher uptake of FA-DADS-SLNs than DADS-SLNs in MCF-7 cells clearly indicates the vital link between
folate receptor over-expression and uptake of folic acid conjugated nanoparticles. These evidences have demonstrated the enhanced cellular uptake of FA-DADS-SLNs as compared with DADS-SLNs.

Apoptosis is a crucial regulatory mechanism of physiological growth and regulation of tissue by terminating superfluous or unwanted cells. This regulatory mechanism is interrupted in cancerous cells and lead to enormous multiplication of cells. Moreover, triggering of apoptosis is a cardinal target for cancer therapy (93). Apoptosis is regulated by several cell signaling pathways. In intrinsic pathway, the cell death occurs itself as it sensitizes the stress conditions whereas extrinsic pathway is associated with the signals from external source. Induction of these pathways triggers activation of several enzymes and proteins such as caspases and proteases. Initiator caspases/proteases activate executioner caspases/proteases and cause the cell death (97). Annexin V/propidium iodide dual staining assay quantified the apoptosis of FA-DADS-SLNs, DADS-SLNs and DADS in MCF-7 cell lines. It was measured in the terms of proportion obtained in gated events and cell death was classified in the four quadrants. The proportion of early apoptotic and late apoptotic cells in control (0.6% and 0.9%), DADS (1.49% and 14.72%), DADS-SLNs (3.2 % and 54.2 %) and FA-DADS-SLNs (5.7 % and 61.8%) was determined. This assay had confirmed the elevated apoptosis by the FA-DADS-SLNs which might be attributed by folate receptor mediated delivery of DADS, sustained drug release from lipid shell and enhanced cellular uptake.

In intrinsic pathway, activation of caspases (initiator caspase-9 and effector caspase-3) is linked with mitochondrial mediator cytochrome-c. Cytochrome c relocation to cytosol is controlled by the pro-apoptotic proteins (Bax, Bad and Bid) and anti-apoptotic proteins (Bcl-2, Bcl-xL) (92). Caspase-3, a cysteine protease is activated by apoptotic signals from both death receptor and intracellular/mitochondrial pathways. Caspase-3 down regulation is associated with breast carcinogenesis because it is the principal effector protease induces cell death by cleaving several cell death substrates (98). In the present investigation effect of DADS encapsulated in FA-DADS-SLNs, DADS-SLNs and naïve DADS on mitochondrial mediated pathway was studied. Western blot analysis revealed that FA-DADS-SLNs treated MCF-7 cell lines exhibited up-regulation of BAX, BAD, Caspase-3, -9 along with the down regulation of BCI-2. These protein expression findings of FA-DADS-SLNs were clearly depicted in molecular level by western blot analysis. In particular, FA-DADS-SLNs exhibited greater efficacy by the site specific drug delivery. But
enhanced action of DADS encapsulated in folic acid triggered higher apoptosis response. Current chemotherapeutics in clinical and pre-clinical trials need targeted delivery to treat cancer cells which can reduce several toxicities. On the whole, Folic acid conjugated drug delivery system exhibited superior targeting action over DADS-SLNs, DADS.

FA-DADS-SLNs can be a suitable tumor targeted drug delivery system as it characterized by nano-sized, sustained release behavior, enhanced cytotoxicity by the folate receptor targeting. In future studies, a detailed investigation need to be carried out to identify the impact on the bio-distribution and tumor regression characteristics of FA-DADS-SLNs using in vivo animal models.