Chapter 7

Preparation and characterization of lupeol loaded SLN

7.1.Introduction

Lupeol is a pentacyclic triterpenoid and it is found in most of the plants. It is pharmacologically active and it has several medicinal values such as antimicrobial activity, anticancer activity, anti-inflammatory activity, antidiabetic activity and it is found to be effective as a contraceptive (Wal et al. 2015). Lupeol is a marker compound present in *Ficus religiosa* L. extract and the effectiveness of lupeol against oxidative stress induced diabetes was studied in the present study. The optimized conditions applied for obtaining smaller particle size, narrow PDI, higher zeta potential and entrapment efficiency and sustained drug release of *Ficus religiosa* L. extract loaded SLN (discussed under section. 6.3.1) were used to prepare lupeol loaded SLN (both targeted SLN, LTNPs and untargeted SLN, LUNPs were prepared. SLN characterization such as particle size, PDI, zeta potential, entrapment efficiency, *in vitro* drug release, *in vitro* cytotoxicity assessment, mitochondrial function assessment *in vivo,* antidiabetic activity, pharmacokinetic assessment (a separate formulation of lupeol loaded SLN without any functionalization), and histology studies was done.

7.2.Methods

7.2.1. Preparation of SLN

SLNs were prepared and characterized by the methods already discussed under section 6.2.

7.3. Results and discussion

7.3.1. *In vitro* characterization of SLN

Slight increase in particle size and PDI was observed between LTNPs and LUNPs. In case of zeta potential, significant difference between targeted and untargeted nanoparticles was observed (Table 7.1). Targeted nanoparticles resulted in zeta potential value of + 58.12 for LTNPs and -37.40 for LTNPs, respectively. This might be due to the positive charge associated with triphenylphosphonium, a mitochondrial targeting moiety used for functionalizing SLN. SEM morphology of LTNPs revealed spherical shape of nanoparticles Figure 7.1A. *In vitro* release profile of plain lupeol in pH 1.2 showed 64.21% release of lupeol in 2 hours and LTNPs and LUNPs showed 30.40% an 35.12%, respectively in pH 1.2 at 2 hours and in pH 6.8, sustained drug release was observed for both LUNPs and LTNPs whereas plain lupeol showed 87.12 % at 4 hours (Figure 7.1B). *In vitro* release profile of lupeol in pH 7.4 showed 92.34%, 58.79% and 59.83% for plain lupeol, LTNPs and LUNPs, respectively (Figure 7.1C). No difference in drug release was observed between LTNPs or LUNPs.

Table 7.1: Particle size, PDI, zeta potential and entrapment efficiency values of LTNPs and LUNPs

Observations	LTNPs	LUNPs
Particle size (nm)	227 ± 49.91	210.35 ± 63.23
PDI	0.35 ± 0.05	0.31 ± 0.09
Zeta potential (mV)	58.12 ± 8.61	-37.40 ± 9.42[b]
EE (%)	57.32 ± 6.54	54.82 ± 7.66

Mean ± SD (n=3)

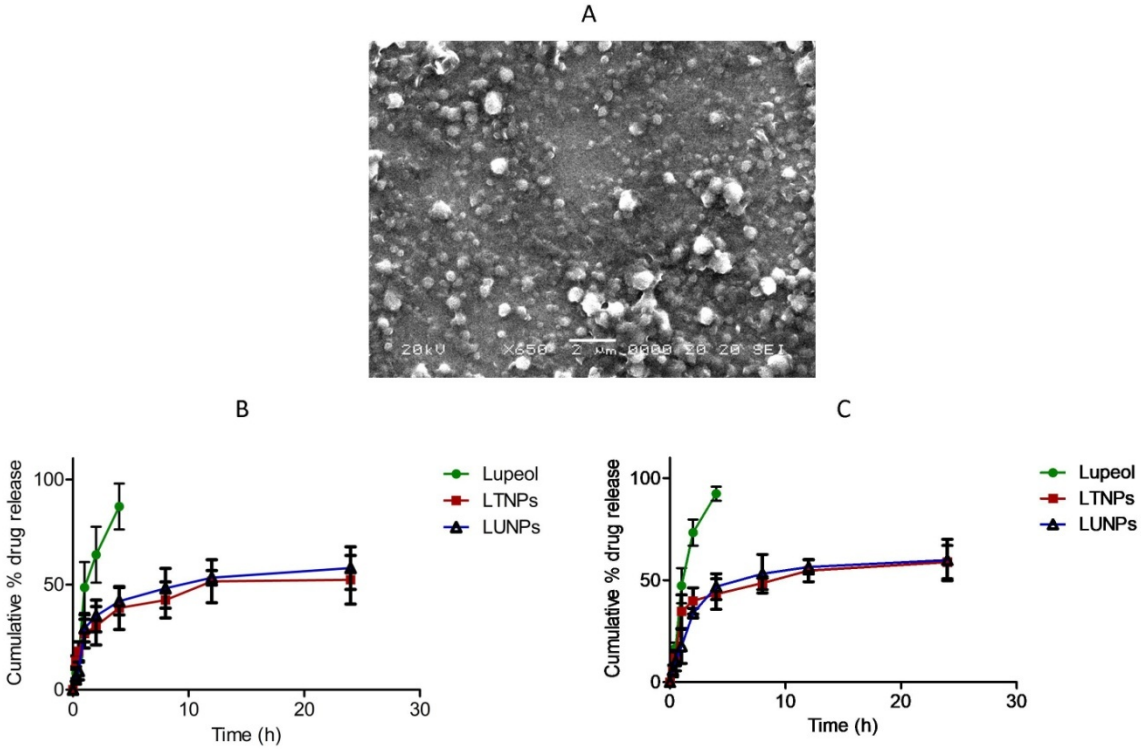

Figure 7.1: A) SEM image of LTNPs and B) i*n vitro* release profiles of lupeol, LUNPs and LTNPs in pH 1.2 for first two hours followed by pH 6.8 and C)) i*n vitro* release profiles of lupeol, LUNPs and LTNPs in pH 7.4.

7.3.2. Interaction studies

From the results of FTIR (Figure 7.2 A), it was observed that lupeol showed characteristic peaks at 3398 (O-H stretching), 2926 and 1383 (C-O bond vibrations), (C-H stretching), 1629 (-C=C- vibration), 1157 (C-N stretching) and 898 (-C=C-H stretching). All these peaks are characteristic peaks of lupeol and all were present in SLN and there was no absence of any functional peaks in all the spectra. Thus, it revealed that there was no significant physicochemical interaction between drug and lipid. DSC studies revealed that in the thermograms of SLNs, peak corresponding to lupeol was reduced and broadened but no change was observed in the lipid peak (Figure 7.2 B). The broadening of lupeol peak in nanoparticles might be due to conversion of crystalline form to amorphous form. PXRD spectra of lupeol, glyceryl monostearate and SLNs are shown in Figure 7.2C. The diffraction spectrum of lupeol showed characteristic peaks at 2θ of 13.64, 14.74, 15.97, 19.37, 21.20, 21.57, 22.90, and 24.32 indicating crystalline nature of the lupeol. The crystalline peaks of lupeol were absent in SLN formulations indicating that the lupeol was not in crystalline form. Intensity of glyceryl monostearate was also decreased in the SLN formulation. This reduced intensity confirms the decreased crystallinity of lipids in SLN formulations.

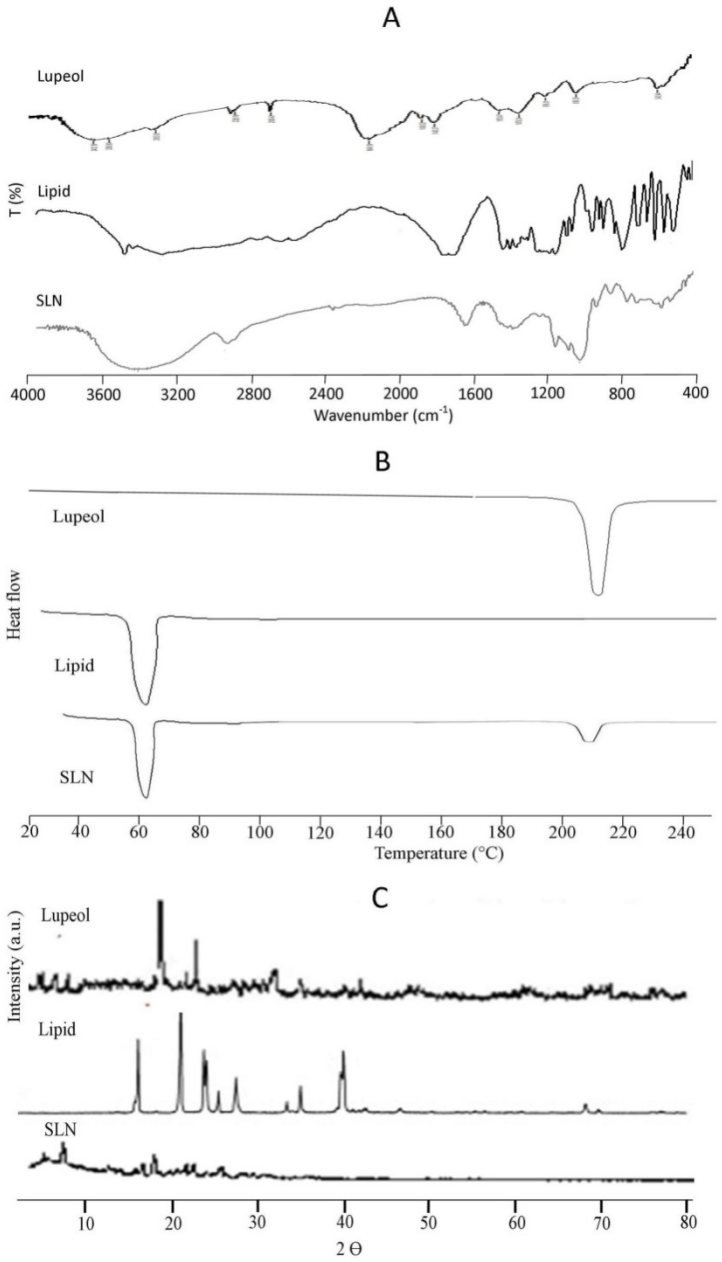

Figure 7.2 : Interaction studies of lupeol, glyceryl monostearate and in SLN form A) FTIR, B) DSC and C) PXRD

7.3.3. *In vitro* cytotoxicity assessment

The results of *in vitro* cytotoxicity assay (Figure 7.3.) revealed that plain lupeol treated cells showed least % cell viability (64 %) which shows the cytotoxic nature of lupeol. However the nano-form of lupeol, LTNPs and LUNPs showed higher % cell

viability due to the surface coating of surfactant and lipid used incorporated during preparation of nanoparticles.

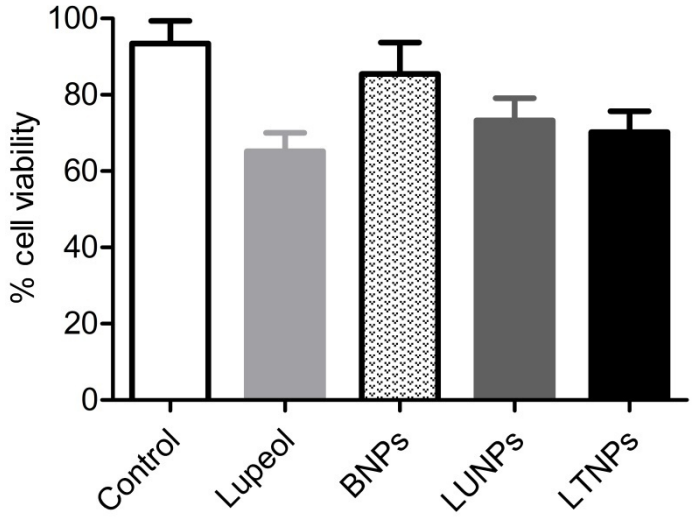

Figure 7.3: *In vitro* cytotoxicity assessments of different treatment groups

7.3.5. *In vivo* results

7.3.5.1.Mitochondrial morphology

The changes in mitochondrial morphology in normal, diabetic condition followed by lupeol, LUNPs and LTNPs treatment groups were studied and the results showed that similar mitochondrion morphology (oval shape and spherical shape) was observed with diabetic, lupeol and LUNPs treated groups which indicated no effect of lupeol and LUNPs treatment on mitochondrial morphology (Figure 7.4). However, treatment with LTNPs had a positive effect on mitochondrion, oval shaped mitochondrion with size similar to that of the control group. This might be due to the targeted effect of lupeol loaded nanoparticles in mitochondria. The images were captured on average basis.

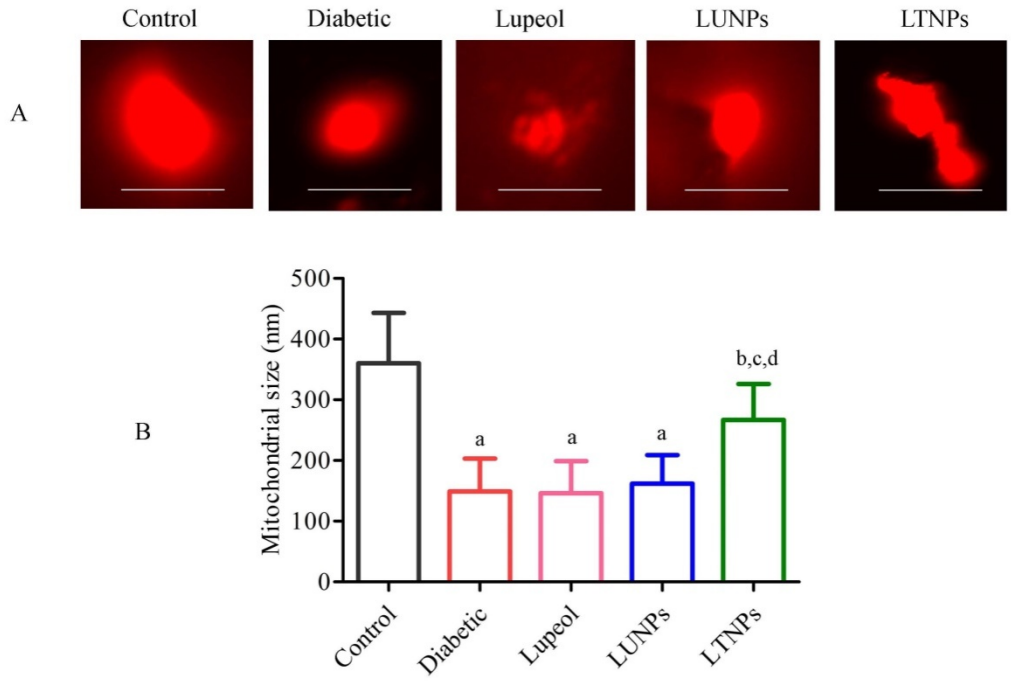

Figure 7.4: Mitochondrial morphological changes: A) representative mitochondrial images for size and shape followed by different treatments; JC-1 was used for staining mitochondria and B) histogram representing the size of mitochondria in different treatment groups

Results are expressed as mean ± SEM (n=6) $^{a}P<$ 0.05 compared to normal group; $^{b}P<$ 0.05 compared to diabetic group;$^{c}P<$ 0.05 compared to lupeol; $^{d}P<$ 0.05 compared to LUNPs (one-way ANOVA followed by Tukey's multiple comparison test).

7.3.5.2.Mitochondrial membrane potential

The changes in mitochondrial membrane potential in normal, diabetic and different treatment groups (lupeol, LUNPs and LTNPs) are shown in Figure 7.5. From the results, it was observed that mitochondrial integrity was lost in diabetic rats than control rats. Mitochondrial integrity was effectively regained in rats treated with lupeol, LUNPs and LTNPs but the effect was significantly ($p<0.05$) different from the control group.

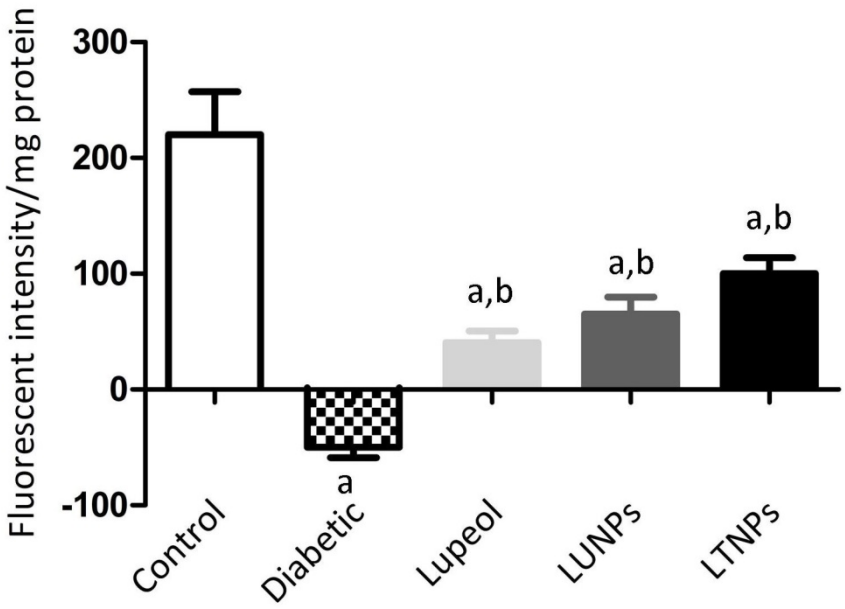

Figure 7.5: Mitochondrial membrane potential of different treatment groups

Results are expressed as mean ± SEM (n=6) [a]P< 0.05 compared to normal group and [b]P< 0.05 compared to diabetic group (one-way ANOVA followed by Tukey's multiple comparison test).

7.3.5.3.Measurement of complex-I, II, IV and V analysis

The function of mitochondrial respiratory chain was assessed by determining the activity of complexes responsible for electron transport chain, complex-I, II, IV and V. The activity of all four complexes was significantly ($p<0.05$) reduced in diabetic group as compared to control group. Treatment with lupeol or LUNPs or LTNPs had no effect on improving the activity of complex-I and IV but treatment with LTNPs significantly ($p<0.05$) improved complex-II and V activities and it was not significantly ($p >0.05$) different form control group (Figure 7.6). This shows the ineffectiveness of LTNPs on improving the different complexes of mitochondrial respiratory chain.

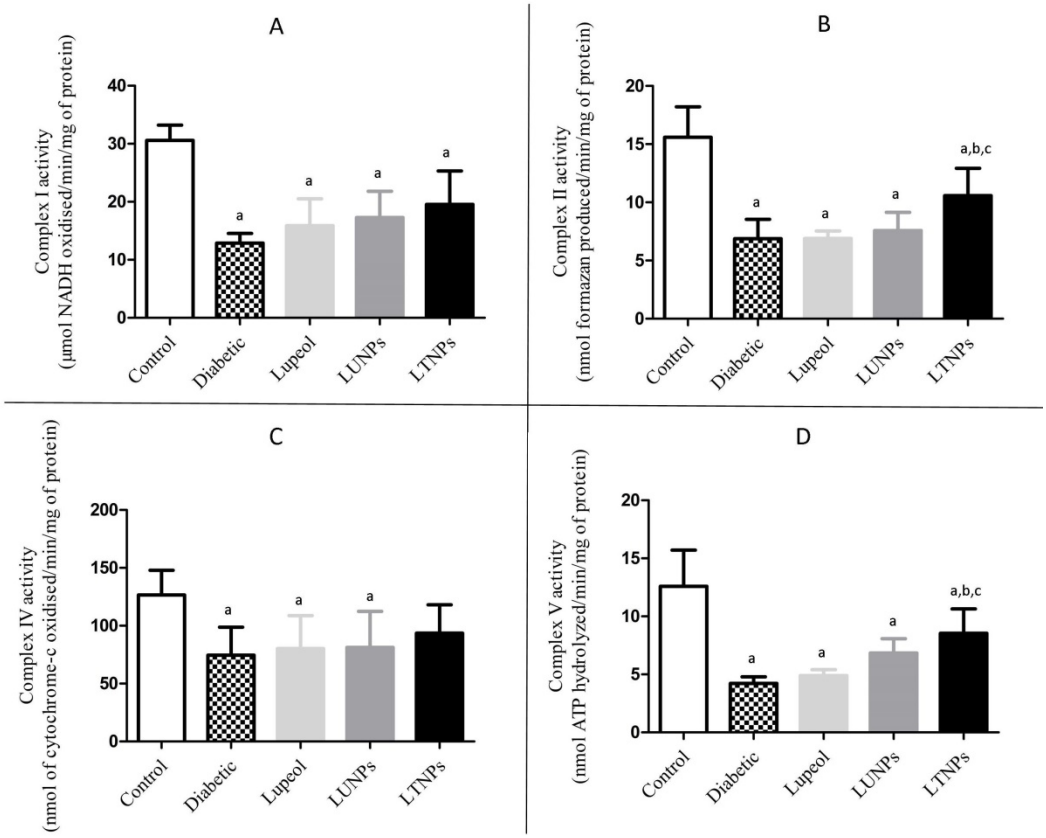

Figure 7.6: Complex-I (A), II (B), IV (C) and V (D) activities of different treatment groups

Results are expressed as mean ± SEM (n=6) $^{a}P<$ 0.05 compared to normal group; $^{b}P<0.05$ compared to diabetic group and $^{c}P<$ 0.05 compared to lupeol (one-way ANOVA followed by Tukey's multiple comparison test).

7.3.5.4.Calcium ion concentration

Quantitative analysis of intracellular calcium ion content (Figure 7.7) revealed significant increase in calcium ion concentration of diabetic group than control group ($p < 0.05$). LTNPs treated group significantly ($p > 0.05$) reversed calcium ion concentration ($p < 0.05$) as compared to LUNPs and lupeol treated groups and the effect was not significantly ($p<0.05$) different from the control group.

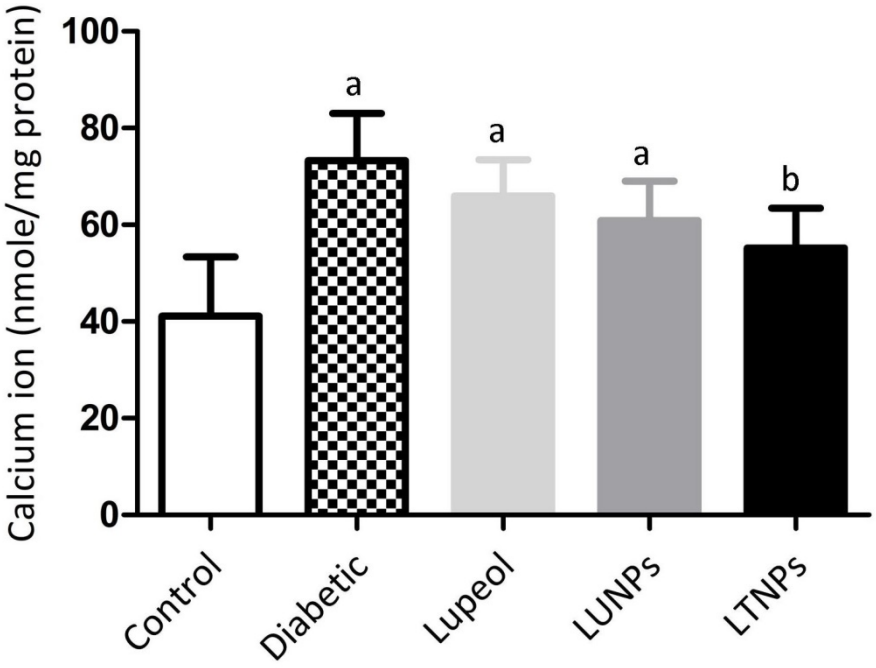

Figure 7.7: Quantitation of calcium ion concentration in different treatment groups

Results are expressed as mean ± SEM (n=6) [a]P< 0.05 compared to normal group and [b]P< 0.05 compared to diabetic group (one-way ANOVA followed by Tukey's multiple comparison test).

7.3.5.5.Western blotting

The expression of apoptotic makers such as cytochrome C, caspase-3 and caspase-9 were studied in diabetic condition followed by different treatment groups (Figure 7.8). All three markers expressions were high in diabetic condition and lupeol and LUNPs treated group showed no significant ($p>0.05$) difference in expression of these markers as compared to diabetes. But significant ($p<0.05$) difference in the expression of caspase-9 and caspase-3 was observed in LTNPs treated groups and LTNPs were failed to reduce the expression of cytochrome C.

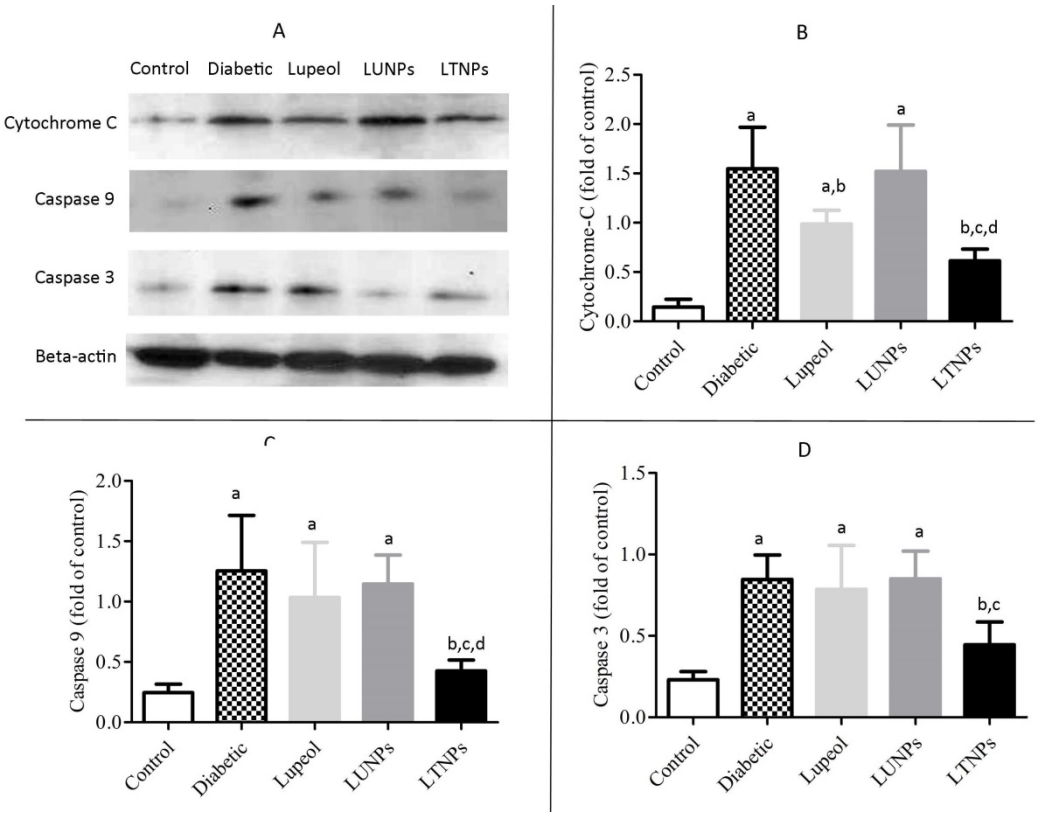

Figure 7.8: A) Western blotting expressions of cytochrome C, caspase-9 and caspase-3, B) intensity of cytochrome C) intensity of caspase-9 and intensity of caspase-3

Results are expressed as mean ± SEM (n=6) [a]P< 0.05 compared to normal group; [b]P< 0.05 compared to diabetic group; [c]P< 0.05 compared to *Ficus religiosa L.* extract; and [d]P< 0.05 compared to EUNPs (one-way ANOVA followed by Tukey's multiple comparison test).

7.3.5.6.ROS levels estimation

From the results (Figure 7.9), it was observed that higher generation of ROS in diabetic group as compared to control and the lupeol had no significant (p>0.05) effect on reduction in ROS generation. However, LTNPs treatment had significant (p<0.05) effect in reducing the ROS generation than LUNPs or lupoel and this could be due to targeted delivery of an lupeol to mitochondria.

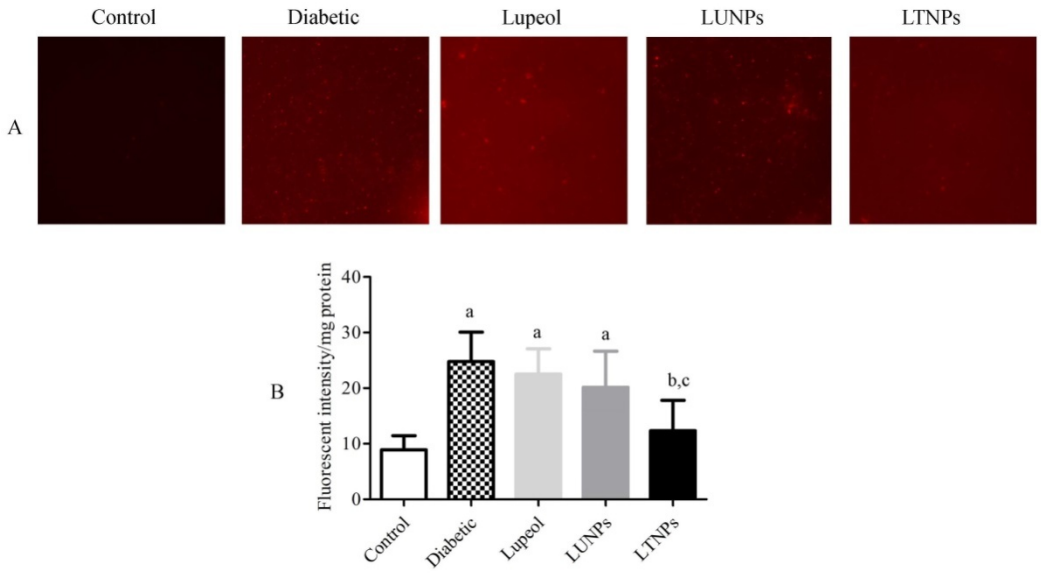

Figure 7.9: ROS generation in different groups A) representative fluorescence microscopic images for ROS generation followed by different treatments and B) histogram representing the fluorescent intensity in different treatment groups

Results are expressed as mean ± SEM (n=6) $^{a}P<$ 0.05 compared to normal group; $^{b}P<$ 0.05 compared to diabetic group; and $^{c}P<$ 0.05 compared to lupeol (one-way ANOVA followed by Tukey's multiple comparison test).

7.3.5.7.Antioxidant enzyme levels

The levels of different antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase were estimated and the results are illustrated in Figure 7.10. It was observed that the levels of superoxide dismutase in diabetic rats were significantly decreased (p>0.05) as compared to control rats (Figure 7.10A). Administration of lupeol or LUNPs or LTNPs did not show any effect on superoxide dismutase level. Similarly, reduced level of catalase was observed in diabetic rats as compared to control rats and different treatment groups had no effect on reversing the catalase levels (Figure 7.10B). In line with earlier observations, decreased level of

glutathione peroxidase was observed in diabetic rats as compared to control rats (Figure 7.10C). Interestingly, treatment with LTNPs significantly ($p<0.05$) reversed the level of glutathione peroxidase as compared to diabetic group.

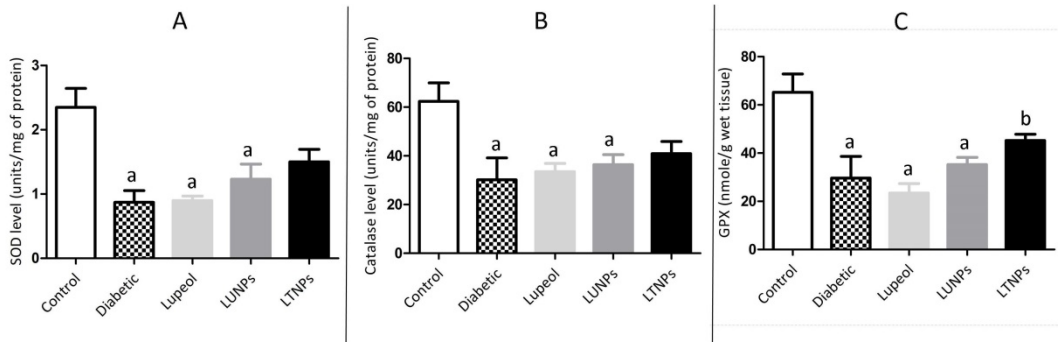

Figure 7.10: Antioxidant levels of different treatment groups on A) superoxide dismutase, B) catalase and C) glutathione peroxidase

Results are expressed as mean ± SEM (n=6) $^{a}P< 0.05$compared to normal group and$^{b}P<$diabetic (one-way ANOVA followed by Tukey's multiple comparison test).

7.3.5.8.Nitrite and malondialdehyde levels

The increased levels of nitrite and malondialdehyde levels in diabetic condition were significantly ($p<0.05$) reversed by LTNPs treatment as compared to diabetic group and plain lupeol or LUNPs had no effect on malondialdehyde levels but LUNPs significantly ($p<0.05$) reduced nitrite level as compared to diabetic group (Figure 7.11).

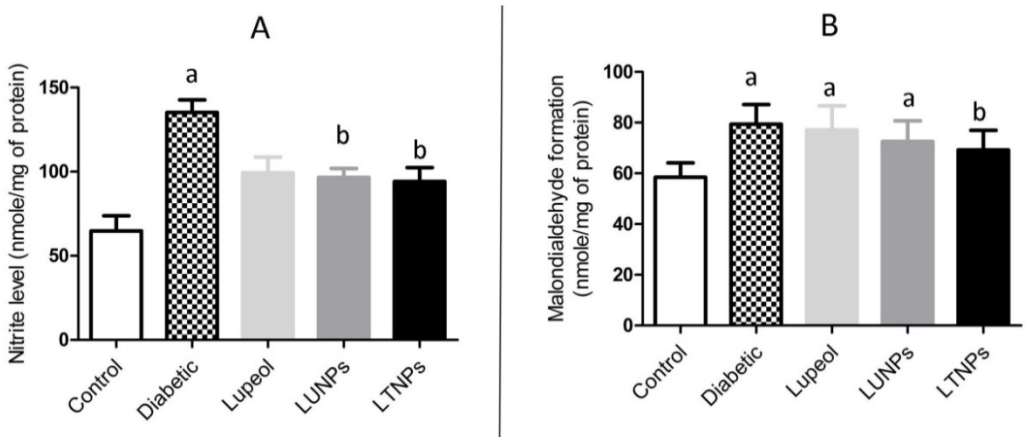

Figure 7.11: Estimation of A) nitrite levels and B) maldialdehyde formation following different treatments

Results are expressed as mean ± SEM (n=6) [a]P< 0.05compared to normal group and [b]P< 0.05 compared to diabetic group(one-way ANOVA followed by Tukey's multiple comparison test).

7.3.5.9.**Diabetic markers analyses**

Effect of lupeol, LUNPs and LTNPs on blood glucose, plasma insulin and serum glycated haemoglobin (HbA1C) levels is shown in Figure 7.12. Results showed increased blood glucose levels in diabetic group than normal group whereas significant reduction ($p<0.05$) in blood glucose level was observed with group treated with LTNPs (Figure 7.12A). Plasma insulin level was found to decrease in diabetic group compared to normal group. Significant improvement ($p<0.05$) in insulin level was effectively achieved by LTNPs as compared to diabetic group (Figure 7.12 B). Further, from the results of glycated haemoglobin levels, it was observed that the level of HbA1C was significantly ($p<0.05$) increased in diabetic group compared to control group as shown in Figure 7.12 C. Treatment with LTNPs significantly reduced HbA1C level ($P<0.05$) whereas treatment with lupeol or UNPs did not have any effect on blood glucose, plasma insulin and glycated hemoglobin levels.

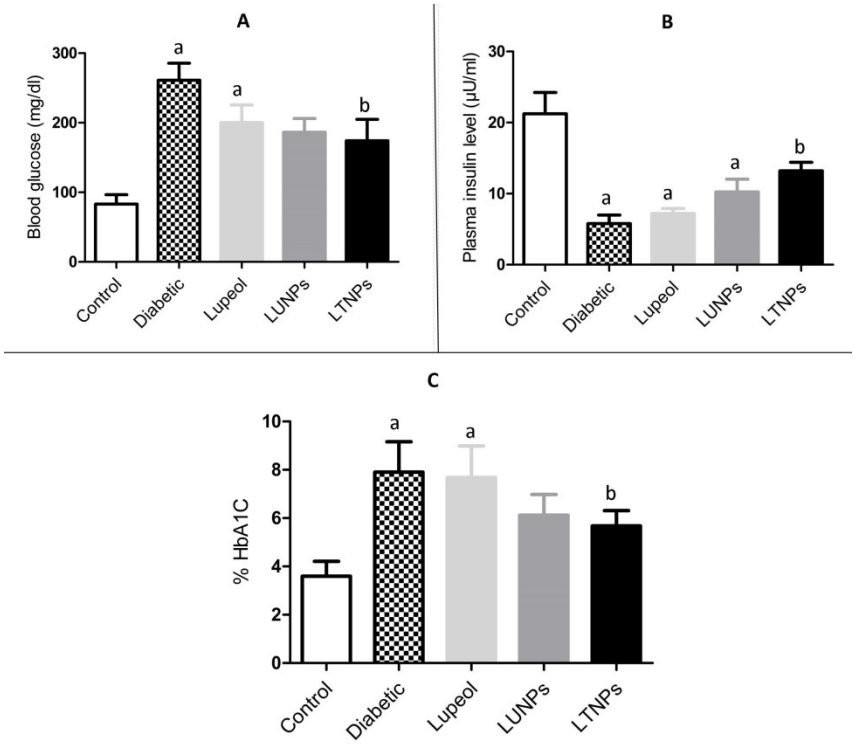

Figure 7.12: Effect of different treatments on A) blood glucose, B) plasma insulin, C) serum glycated haemoglobin

Results are expressed as mean ± SEM (n=6) $^{a}P< 0.05$ compared to normal group and $^{b}P< 0.05$ compared to diabetic group (one-way ANOVA followed by Tukey's multiple comparison test).

7.3.5.10. Histopathology studies

The damage observed in the cells of pancreas, liver, skeletal muscle, adipose tissue and kidney in diabetic condition was effectively treated by LTNPs as shown in Figure 7.13. This can be due to the targeted delivery of lupeol in nanoparticles form. Further, the results of histopathology study in different organs suggest that oral administration of LTNPs are safe to use for the effective management of oxidative stress induced diabetes and non-toxic *in vivo*. Regeneration of damaged tissues in all organs studied in diabetic condition was observed in lupeol and LUNPs treatment.

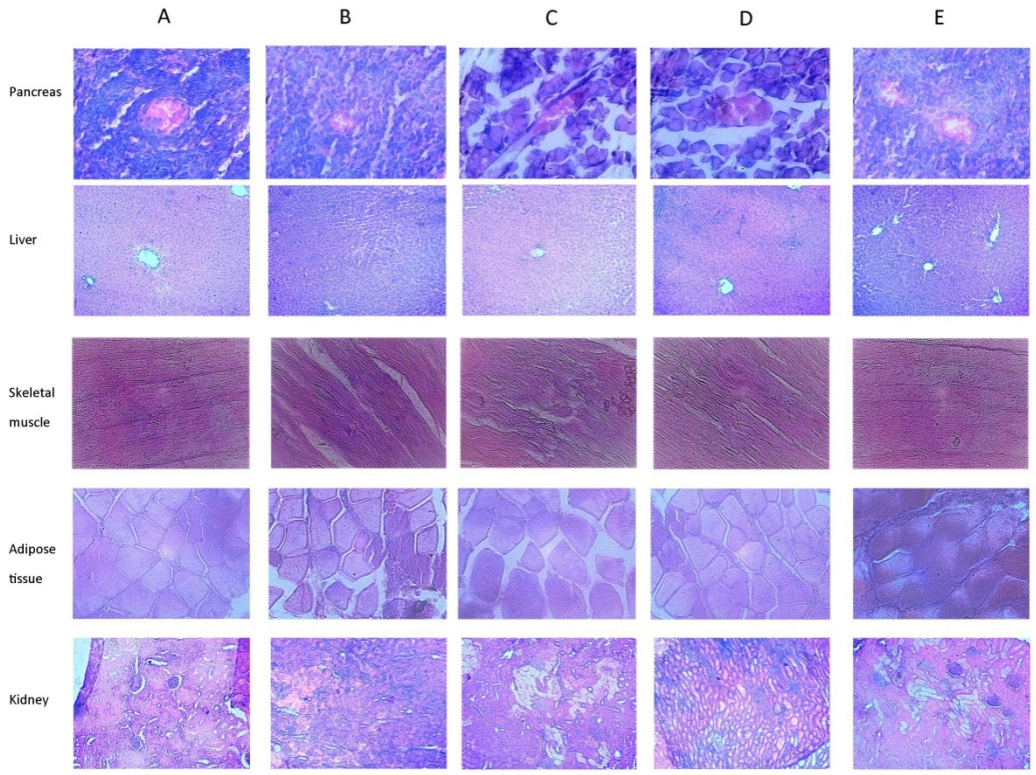

Figure 7.13: Histology examination of different organs followed by different treatments A) control rat, B) diabetic, C) lupeol, D) LUNPs and E) LTNPs

7.3.5.11. Pharmacokinetic assessment

The mean plasma concentration vs. time curve profiles of lupeolin plain lupeol and lupeol loaded SLN are illustrated in Figure 7.14 and pharmacokinetic parameters of lupeol in plain lupeol and lupeol loaded SLN are shown in Table 7.2. The mean plasma AUC_{0-24} of lupeol in animals treated with SLN formulation was 2.6-fold higher than plain lupeol. This increase in AUC_{0-24} for SLN might be due to the avoidance of first pass metabolism by lymphatic transport. The peak plasma concentration (C_{max}) of lupeol in SLN formulation was 3.2-fold higher than lupeol. Time to reach plasma concentration (t_{max}) in plain lupeol was found to be 8 ± 1.3 h and in SLN formulation was found to be 2 ± 0.11 hours. $t_{1/2}$ of lupeolin plain lupeol was 4.6 hours and in SLN formulation was 13.8

± 1.6hours. From these results, it clearly suggested that the pharmacokinetic profiles of lupeol have been improved in SLN form than plain lupeol after oral administration.

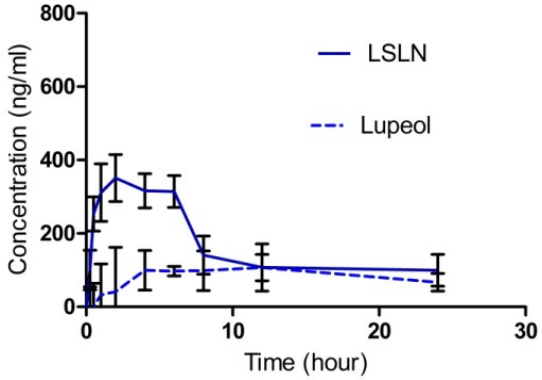

Figure 7.14. Plasma concentration vs. time curve of lupeol in *Ficusreligiosa*Linn.extract suspension and SLN

Table 7.2: Pharmacokinetic parameters of lupeol in *Ficus religiosa* L. in rat plasma

Parameter	Lupeol	LSLN
AUC0-24 (ng × hr/ml)	452.37 ± 114.78[a]	1184 ± 256.80[b]
C_{max}(ng/mL)	107.30[a]	351.00[b]
T_{max}(hr)	8	2[b]
$t_{1/2}$ (h)	4.6 ± 1.0	13.8 ± 1.6[b]

Mean ± SD; n=6

*p< 0.05, significance difference compared to lupeol.

7.4. **Summary**

In this study, lupeol loaded nanoparticles functionalized by using TPP were prepared (LTNPs), and lupeol loaded nanoparticles which were not functionalized by using TPP (LUNPs) were prepared. The efficiency of LUNPs and LTNPs were studied in diabetic condition and compared with plain lupeol. LTNPs were found to be effective in maintaining the mitochondrial integrity but not LUNPs or lupeol. Further, LTNPs reduced the increased levels of blood glucose and glycated hemoglobin and improved plasma insulin levels. The safety of LTNPs was suggested by the histopathological studies. A separate pharmacokinetic study was performed which showed the improved pharmacokinetic parameters of lupeol in SLN form than plain lupeol.