# Chapter 8

# *In vitro* and *in vivo* comparison between ETNPs and LTNPs

## 8.1. **Introduction**

The effect of *Ficus religisosa* L. extract and its marker compound, lupeol and their nano forms on the oxidative stress induced diabetes was studied to evaluate the efficiency of extract and marker compound. Generally, phytochemicals are costlier due to the process involved in separation and purification from the herbal source whereas extracts are relatively cheaper because of their wider availability. However, in terms of pharmacological action, extracts possess more than one pharmacological actions and lesser side effect compared to single phytochemical (Wagner and Ulrich, 2009). Hence, the effect of single phytochemical, lupeol and the source of the phytochemical, *Ficus religiosa* L. extract was studied both pharmacodynamically and pharmacokinetically.

## 8.2. *In vitro* comparison

### 8.2.1. Preparation and characterization of SLN

The methods involved in the preparation and characterization of ETNPs and LTNPs have already been discussed under the section, 6.2.

The results of particle size, PDI, zeta potential and entrapment efficiency of both untargeted and targeted nanoparticles of extract and lupeol are given in Table 8.1. Slight increase in particle size and PDI was observed between targeted and untargeted nanoparticles in both extract- and lupeol- loaded SLN. In case of zeta potential, significant difference between targeted and untargeted nanoparticles was observed. Targeted nanoparticles resulted in zeta potential value of + 57.31 for ETNPs and +58.13 for LTNPs whereas -39.78 and -37.40 for ETNPs and LTNPs, respectively. This might be due to the positive charge associated with triphenylphosphonium, a mitochondrial targeting moiety used for functionalizing SLN. SEM morphology of ETNPs and LTNPs revealed spherical shape of nanoparticles is shown in Figure 8.1 A and B, respectively. *In vitro* release profile of extract in pH 1.2 for the first two hours followed by the use of phosphate buffer, pH 6.8, showed 96.78 % release of lupeol in 4 hours and in case of plain lupeol, the drug release was 88.23 % in 4 hours. The highest drug release of lupeol in extract might be due to the increase solubility of lupeol in extract form. In case of nanoparticles, ETNPs or EUNPs or LTNPs or LUNPs, sustained drug release was observed (Figure 8.1 C) and % drug release was around 56 at 24 hours and no difference between ETNPs or LTNPs was observed.

**Table 8.1. Particle size, PDI, zeta potential and entrapment efficiency of ETNPs, EUNPs, LTNPs and LUNPs**

| Observations | ETNPs | EUNPs | LTNPs | LUNPs |
|---|---|---|---|---|
| Particle size (nm) | 236 ± 63.48 | 190 ± 57.11 | 227 ± 49.91 | 210.35 ± 63.23 |
| PDI | 0.38 ± 0.02 | 0.30 ± 0.07 | 0.35 ± 0.05 | 0.31 ± 0.09 |
| Zeta potential (mV) | 57.31 ± 7.65 | -39.78 ± 6.87[a] | 58.12 ± 8.61 | -37.40 ± 9.42[b] |
| EE (%) | 59.14 ± 7.19 | 58.72 ± 4.90 | 57.32 ± 6.54 | 54.82 ± 7.66 |

Mean values ± SD; n=3 [a] $P< 0.05$ compared to ETNPs and [b] $P< 0.05$ compared to LTNPs Unpaired t-test

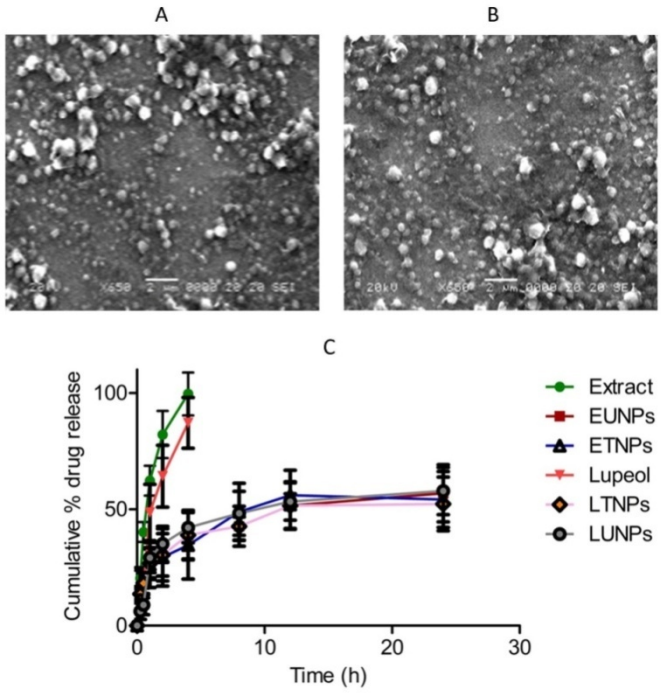


**Figure 8.1.*In vitro* characterization of ETNPs and LTNPs A) SEM image of ETNPs, B) SEM image of LTNPs and C) *in vitro* drug release**

### *8.2.2. In vitro* cytotoxicity assay

The results of *in vitro* cytotoxicity assay (Figure 8.2.) revealed that extract, TNPs (both extract and lupeol) and UNPs (both extract and lupeol) had % cell viability around 80 % which shows that the nanoparticles prepared using GMS, poloxamer 188 and sodium deoxycholate as surfactant and triphenylphosphonium at 1 nmol concentration are safe to use. Of the different treatment groups tested, plain lupeol treated cells showed least % cell viability (64 %) which shows the cytotoxic nature of lupeol. However the nano-form of lupeol, TNPs and UNPs showed higher % cell viability due to the surface coating of surfactant and lipid used incorporated during preparation of nanoparticles. Further, glycerylmonostearate used as a lipid carrier is biocompatible in nature and the surfactants used such as poloxamer 188 and sodium deoxycholate are FDA approved additives.

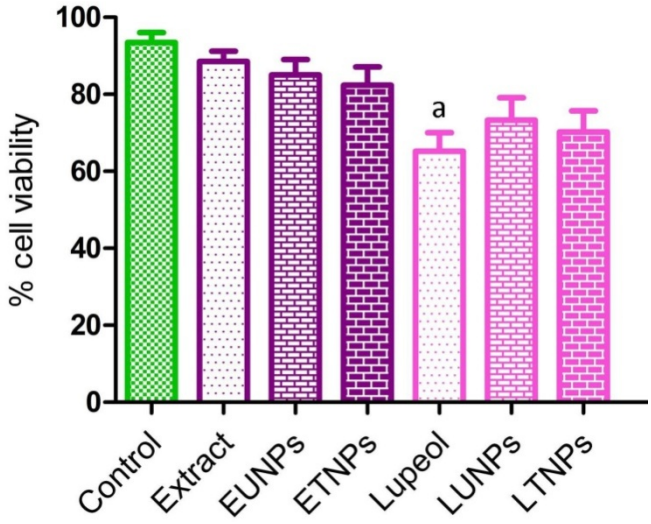


**Figure 8.2.*In vitro* cytotoxicity profile of all treatment groups**

Results are expressed as mean ± SEM (n=3) ($^{a}P< 0.05$ compared to control group; one-way ANOVA followed by Tukey's multiple comparison test).

## *8.3. In vivo* comparison

### 8.3.1. Mitochondrial morphology

The changes in mitochondrial morphology in control, diabetic and different treatment groups were studied and compared. In the control group, mitochondria seem to be in oval shape and the size was found to be in 35 µm whereas in case of diabetic rat, mitochondria size seem to be in spherical shape and the size was less than 25 µm. As suggested by the literature, the shape of mitochondria changes continuously depending on the fission and fusion processes. When there is a more energy requirement of ATP, mitochondria undergoes fission and fusion process and multiplies based on the energy requirement. But lesser size of mitochondria indicate the mitochondrial dysfunction due to lack of mitochondrial proteins. In case of different treatment groups, extract, lupeol or LUNPs the shape and size of mitochondria seem to be in oval and small, respectively as compared to control group (Figure 8.3). However, treatment with ETNPs or LTNPs regained the oval shape of mitochondria and the size was similar to control group suggesting that the targeted nanoparticles have greater impact on the mitochondrial morphology and improved the mitochondrial function. In case of EUNPs treatment, the size and shape of mitochondria were similar to ETNPs or LTNPs group but the fluorescent intensity was lesser than the ETNPs or LTNPs treatment groups.

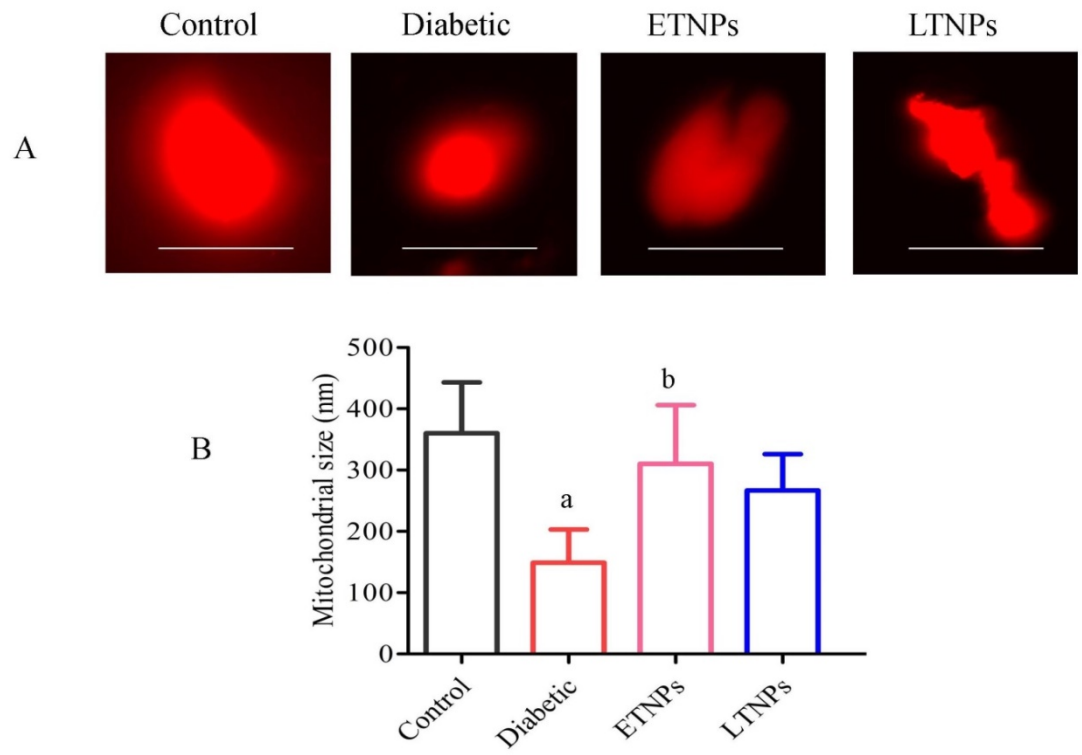


**Figure 8.3.Mitochondrial morphological changes A) representative mitochondrial images for size and shape followed by different treatments; JC-1 was used for staining mitochondria and B) histogram representing the size of mitochondria in different treatment groups**

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

### 8.3.2. Mitochondrial membrane potential

The key indicator of mitochondrial function is the changes in mitochondria membrane potential. In normal condition, the mitochondrial membrane potential ranges between 150 mV and 200 mV. When ROS level is increased, mitochondrial membrane potential decreases to negative value and thus, reduced electron transport capability of electron transport chain. Rhodamine 123 is a fluorescent dye and it specifically stains mitochondria for visualization. Due to its lipophilic and cationic properties, it

accumulates into mitochondria by several folds. In the control group, mitochondrial membrane potential was around 320 mV and in case of diabetes, significant ($p<0.05$) reduction in mitochondrial membrane potential as compared to control rats was observed (Figure 8.4). Treatment with ETNPs significantly ($p<0.05$) regained the mitochondrial integrity than EUNPs or LTNPs or LUNPs. No significant ($p>0.05$) difference was observed between control and ETNPs groups. Plain extract or lupeol had no effect on mitochondrial membrane potential. The targeted delivery of both extract and lupeol as ETNPs and LTNPs, respectively had positive effect of mitochondrial membrane potential.

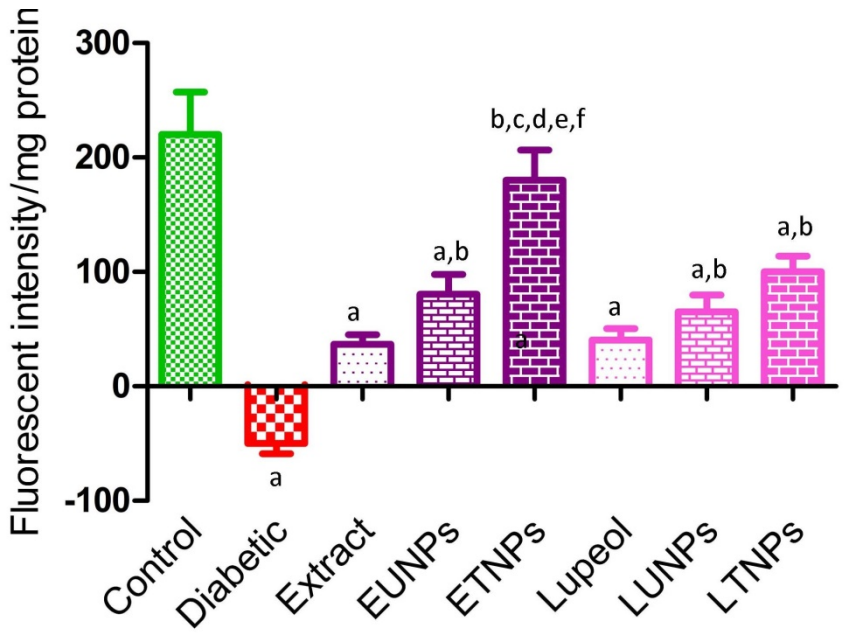


**Figure 8.4.Effect of different treatments on mitochondrial membrane potential**

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 *Ficusreligiosa*L. extract; and [d]$P<0.05$ compared to EUNPs; [e]$P<0.05$ compared to lupeol; [f]$P<0.05$ compared to LUNPs (one-way ANOVA followed by Tukey's multiple comparison test).

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

In ETC, five complexes are involved in the production of ATP. Complex I converts NADH to $NAD^+$, $NAD^+$ is further converted into $FMNH_2$ by flavin mononucleotide and $FMNH_2$ gets oxidized through semiquinone intermediate present in complex I (Weiss, Friedrich et al. 1991). Complex II oxidizes succinate molecules (intermediate of TCA cycle with the use of FAD as coenzyme, three iron-sulfur clusters and cyto-chrome $b_{560}$) into malate and liberates reducing equivalents ($e^-$) that are shuttled to complex III via ubiquinone. In complex III, two electrons received from complex II and are shuttled to complex IV. Complex IV is a transmembrane complex that receives electrons, translocates four protons per pair of electrons and reduces oxygen to water with the liberation of two protons ($H^+$). Liberated protons are utilized by complex V (ATP synthase or $F_1F_0$ ATPase) by proton gradient which creates a transmembrane potential, driving force for the production of ATP from ADP(Lorenzi 2007). Hence, it is necessary to evaluate the function of different complexes responsible for electron transport chain. Of the different treatment groups, ETNPs significantly ($p<0.05$) improved complex-I, II, IV and V activities than LTNPs/EUNPs/LUNPs/extract/lupeol and the activity is similar to that of the control (Figure 8.5). LTNPs significantly ($p<0.05$) improved complex-I and complex-V activities but not complex-II and complex-IV activities. Plain extract or lupeol had no effect on improving complex-I, II, IV and V activity. The improved activity of ETNPs is due to the targeted effect of extract to mitochondria since extract contains several other antioxidant compounds which helps in neutralization of ROS than the LTNPs (as single antioxidant, lupeol is targeted into mitochondria).

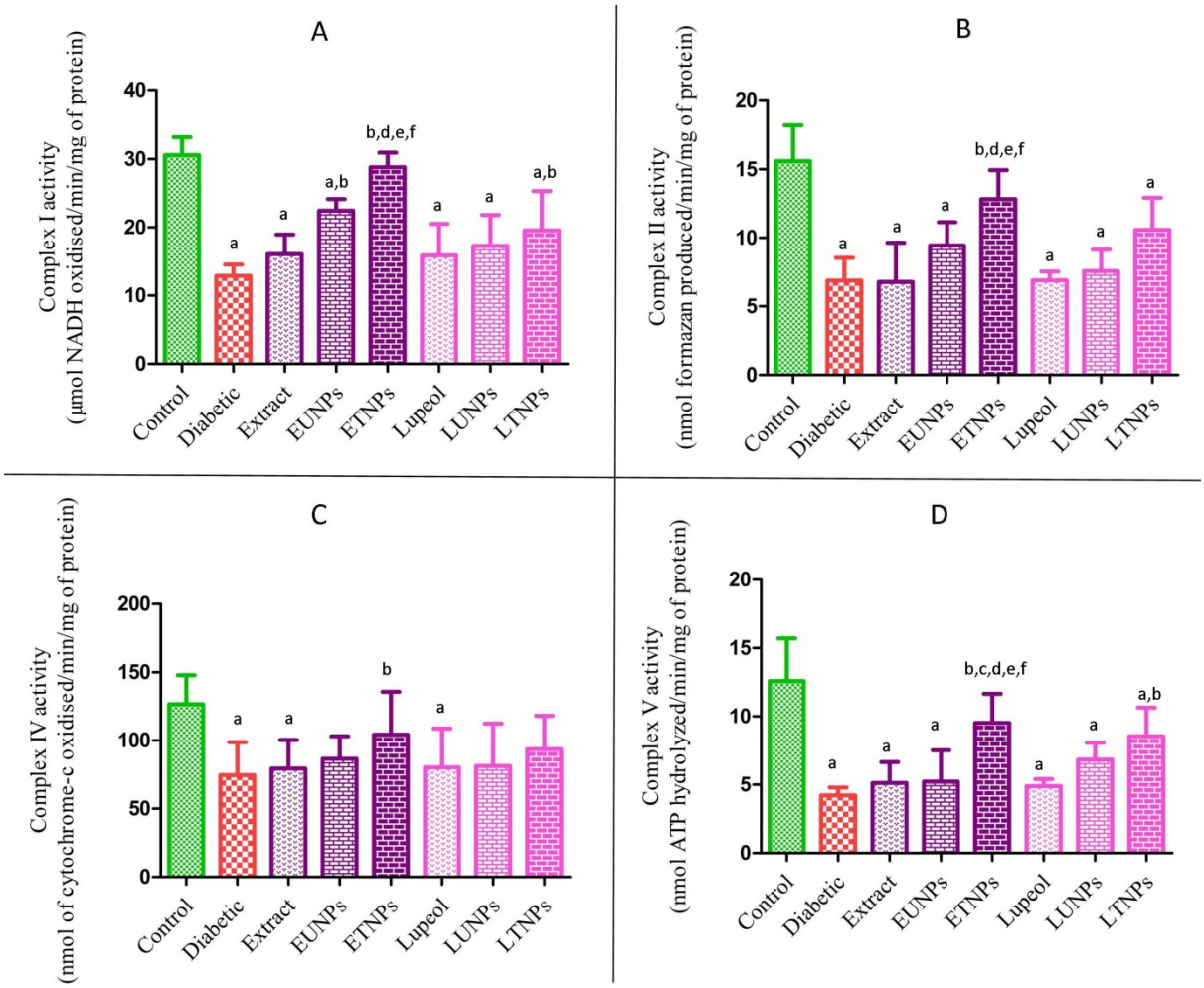


**Figure 8.5.Effect of different treatments on complex-I, II, IV and V activities**

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 *Ficusreligiosa*L. extract; and [d]$P$< 0.05 compared to EUNPs; [e]$P$< 0.05 compared to lupeol; [f]$P$< 0.05 compared to LUNPs (one-way ANOVA followed by Tukey's multiple comparison test).

### 8.3.4. Estimation of calcium ion concentration

Uptake of calcium ion by mitochondria modulates a number of calcium dependent proteins and enzymes which are responsible for tricarboxylic acid cycle and oxidative phosphorylation. Changes in the uptake of calcium ion by mitochondria during

mitochondrial dysfunction result in cellular apoptosis. The levels of calcium ion different treatment group are shown in Figure 8.6. In case of diabetic group, increased calcium ion was observed as compared to control. ETNPs significantly ($p<0.05$) decreased the calcium ion concentration to normal as compared to extract or EUNPs or lupeol or LUNPs or LTNPs and similar to that of control group. This shows that ETNPs had a positive effect in the regulation of calcium ion homeostasis and thus preventing the cellular death.

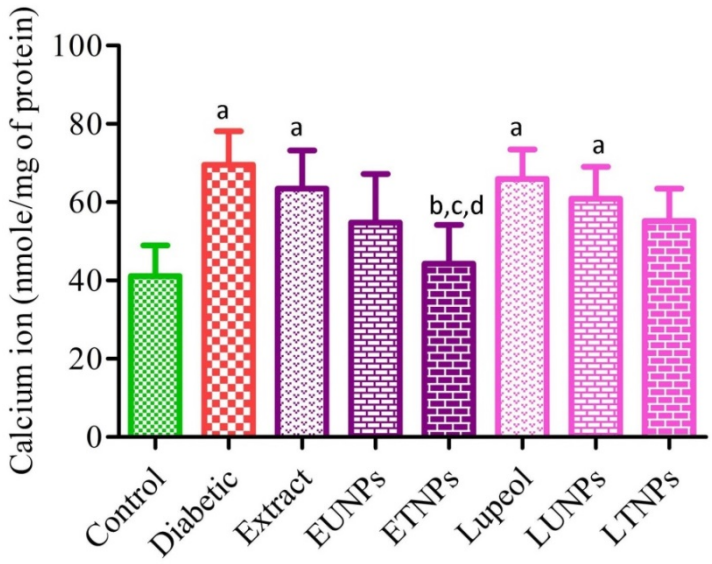


**Figure 8.6.Effect of different treatments on calcium ion levels**

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

### 8.3.5. Western blotting

The apoptotic markers of cell death such as cytochrome C, caspase-9 and caspase-3 were studied and the results suggested that both ETNPs and LTNPs have reduced the expression of

caspase-9 and caspase-9. ETNPs have further reduced the expression of cytochrome C (Figure 8.7) but LTNPs had the cytochrome C expression similar to that of diabetic group which shows that the LTNPs are effective in reducing the cytochrome C level.

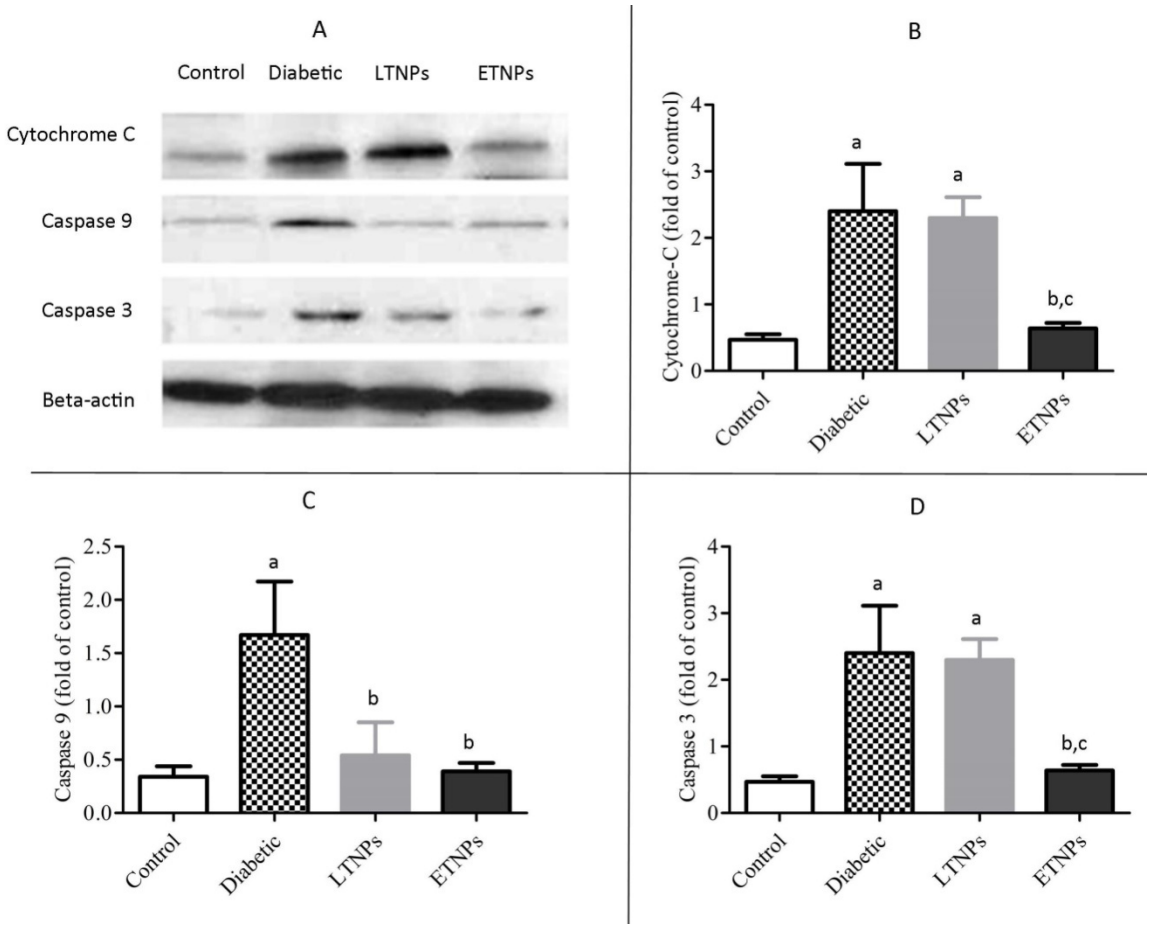


**Figure 8.7: A) Western blotting expressions of cytochrome C, caspase-9 and caspase-3, B) intensity of cytochrome C) intensity of caspase-9 and intensity of caspase-3**

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 *Ficusreligiosa*L. extract; and $^{d}P< 0.05$ compared to EUNPs (one-way ANOVA followed by Tukey's multiple comparison test).

### 8.3.6. ROS levels estimation

The increased ROS level impact on diabetes occurs by several mechanisms such as increased ATP to ADP ratio, generation of 8-hydroxydeoxyguanosine, activation of mitogen-activated protein kinases and increased RNS generation. Of the different groups studied, only ETNPs treated group had significantly ($p<0.05$) reduced ROS level as compared to extract or EUNPs or lupeol or LUNPs or LTNPs (Figure 8.8). This shows the improved antioxidant activity of targeted antioxidant extract to mitochondria.

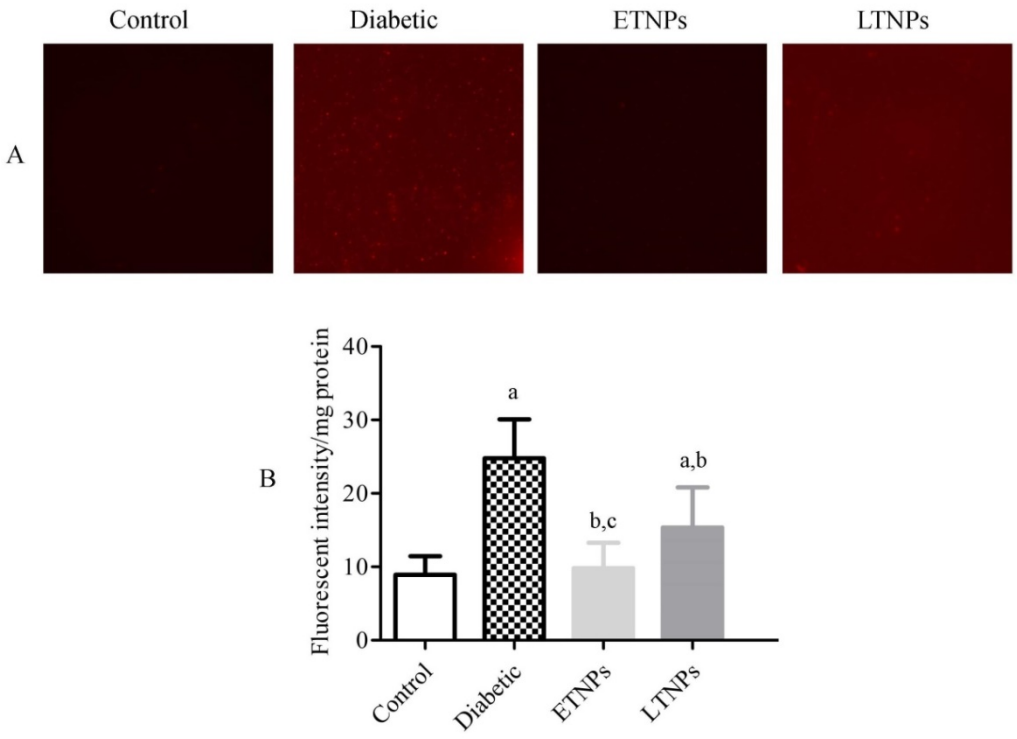


**Figure 8.8:Effect of different treatments on ROS level**

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]$P<0.05$ to LTNPs (one-way ANOVA followed by Tukey's multiple comparison test).

### 8.3.7. Estimation of antioxidant levels

During conversion of glucose to ATP molecules through glycolysis, TCA cycle and ETC pathways, ROS are being produced in the electron transport chain of mitochondria and are neutralized by the anti-oxidant defence mechanism (Addabbo, Montagnani et al. 2009). Normal levels of ROS are necessary for cellular functions including insulin release (Patti and Corvera 2010). When the levels of ROS exceeds the levels of antioxidants in cell for neutralization, neutralization of free radicals will not happen which leads to increased levels of ROS (Rahman 2007). Increased ROS levels play important role in diabetes. Increased ROS level causes insulin resistance in case of β-cells and finally cellular death. Thus, it is mandatory to estimate antioxidant enzyme levels in diabetic condition. Anti-oxidant enzymes responsible for neutralizing ROS include superoxide dismutase, catalase and glutathione peroxidase. The importance of these enzymes in ROS neutralization has already been discussed in the chapter 6, section 6.3.11.7. From the results, it was observed that ETNPs had improved all three antioxidant enzymes and the effect was significantly ($p<0.05$) higher than EUNPs or LTNPs or LUNPs or extract or lupeol. The targeted delivery of ETNPs to mitochondria, make ETNPs to release extract inside the mitochondria and several phytoconstitutents of extract possessing antioxidant activity (lupeol, lanosterol, stigmasterol, sitosterol, kaempferol, quercetin) give electron for ROS neutralization and eventually increasing the antioxidant enzyme levels. In case of LTNPs, even the targeted delivery of lupeol to mitochondria is not enough to produce its antioxidant effect as compared to ETNPs significantly ($p<0.05$). Plain extract or lupeol or EUNPs or LUNPs had no positive effect on antioxidant enzyme levels (Figure 8.9).

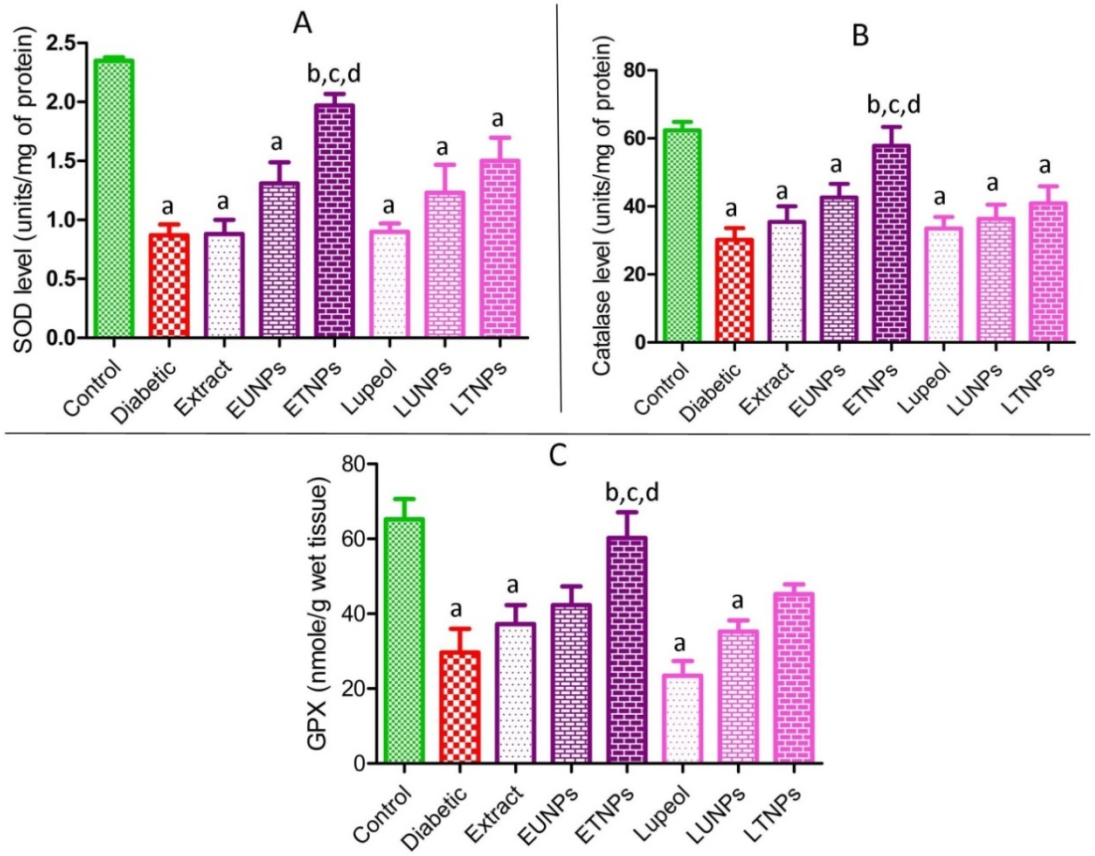


**Figure 8.9:Effect of different treatments on A: superoxide dismutase levels, B: catalase levels, C: glutathione peroxidase levels**

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 extract; and $^{d}P< 0.05$ compared to untargeted nanoparticles (one-way ANOVA followed by Tukey's multiple comparison test).

### 8.3.8. Nitrite and malondialdehyde levels

The increased level of nitrite and malondialdehyde show the impairment in ROS neutralization and they serve as apoptotic markers in the oxidative stress induced diabetes. In the present research, both the levels of nitrite and malondialdehyde were found to be higher as compared to control group (Figure 8.10). In the treatment groups of extract, both EUNPs and ETNPs exerted their action against nitrite by reducing its level. However, ETNPs effect on nitrite level reduction was significantly ($p<0.05$) higher than the EUNPs treatment group

and effect was not significantly ($p>0.05$) than the control group. In case of malondialdehyde levels, only ETNPs significantly ($p<0.05$) reduced the level as compared to diabetic group and all other treatment groups were found to be ineffective in reducing both nitrite and malondialdehyde levels as compared to ETNPs.

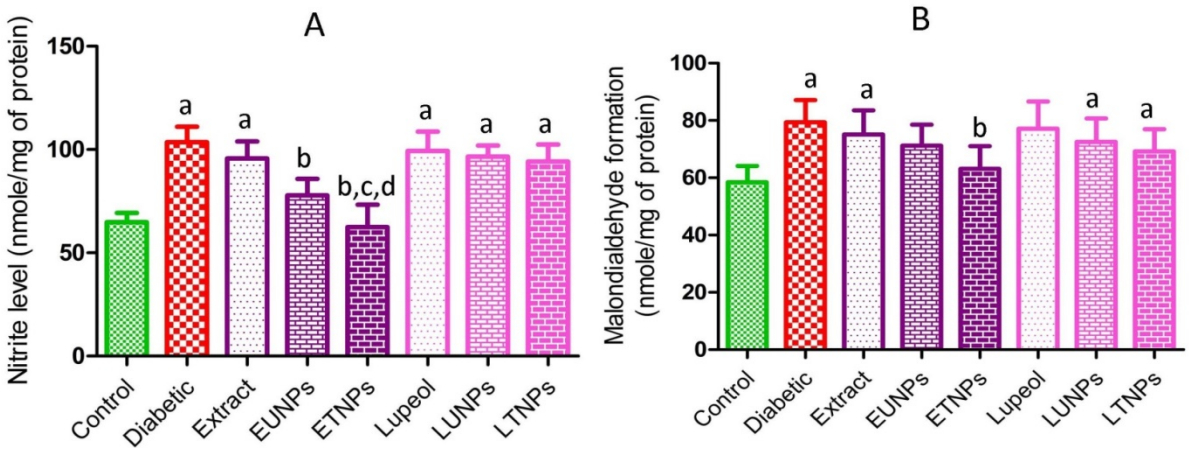


**Figure 8.10: Effect of different treatments on A: nitrite levels and B: malondialdehyde levels**

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 extract; and $^{d}P< 0.05$ compared to untargeted nanoparticles (one-way ANOVA followed by Tukey's multiple comparison test).

### 8.3.9. Diabetic markers analyses

Diabetic markers such as blood glucose, plasma insulin and glycatedhemoglobin were studied and the effect of different treatment on these biomarkers is shown in Figure 8.11. There was an significant ($p<0.05$) increase in blood glucose and glycatedhemoglobin levels was observed in diabetic group as compared to control group and significant ($p<0.05$) reduction in plasma insulin levels was observed in diabetic group

as compared to control group. ETNPs had a significant ($p<0.05$) effect in the reduction of blood glucose and glycatedhemoglobin levels and in improving the levels of plasma insulin than EUNPs or LTNPs or LUNPs or lupeol or extract treated groups. This can be correlated with the reduced ROS level and improved mitochondria function in diabetic rats treated with ETNPs and the mixed pharmacological effect of phytoconstituents present in extract at the target site, mitochondria. Further, this supports that the targeted delivery of extract loaded nanoparticles is effective in the management of oxidative stress induced diabetes. Further, the effect of ETNPs was compared with the marketed product of metformin, Glumet®. Metformin is used as model due to the reason that it selectively targets complex I of the ETC and controls redox system (Wheaton et al. 2014). Results showed that ETNPs have the similar antidiabetic efficiency in controlling the blood glucose and glycated haemoglobin levels and improve plasma insulin level as that of the metformin which proves that ETNPs can be used as replacement of metformin.

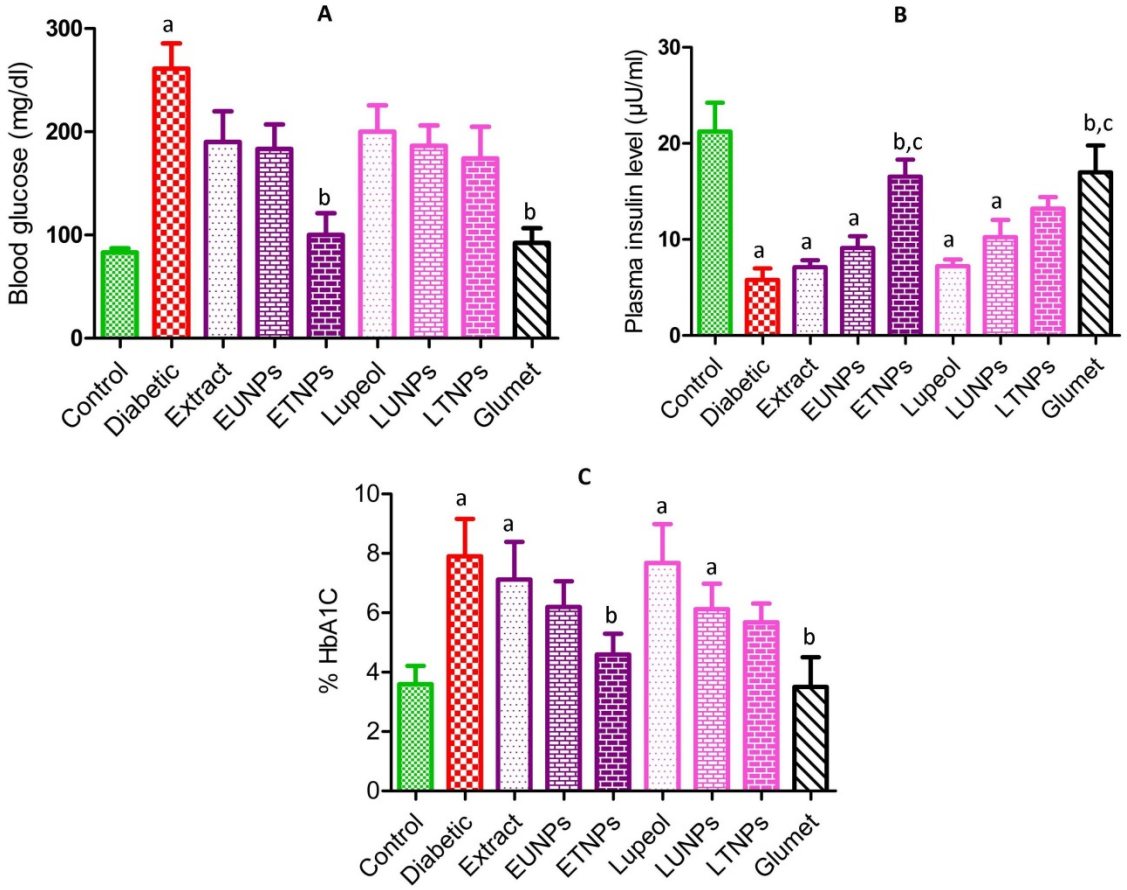


**Figure 8.11: Effect of different treatments on A) blood glucose, B) plasma insulin and C) glycated hemoglobin levels**

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 extract (one-way ANOVA followed by Tukey's multiple comparison test).

### 8.3.10. Histopathology studies

The damage observed in the cells of pancreas, liver, skeletal muscle, adipose tissue and kidney was effectively treated by both ETNPs and LTNPs as shown in Figure 8.12. This can be due to the targeted delivery of extract and lupeol in nanoparticles form. Further, the histopathology study of different organs followed by the oral administration of ETNPs or LTNPs are safe to use for the effective management of oxidative stress induced diabetes and non-toxic *in vivo*.

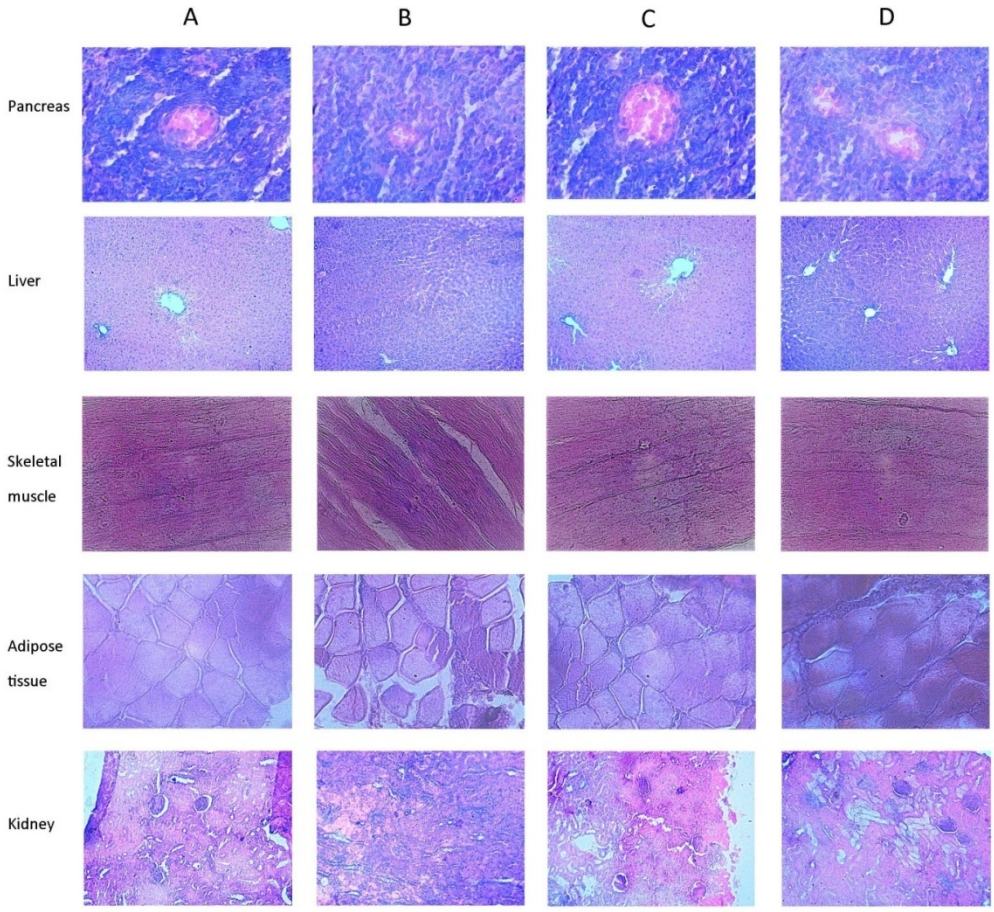


**Figure 8.12: Histology examination of different organs followed by different treatments A) control rat, B) diabetic, C) ETNPs and D) LTNPs**

### 8.3.11. Pharmacokinetic studies

The pharmacokinetic profile of lupeol in plain lupeol, plain extract, LTNPs and ETNPs is shown in Figure 8.13 and Table 8.2. The mean plasma $AUC_{0-24}$ of lupeol in animals treated with *Ficusreligosa*L. extract loaded SLN (ESLN) was 9829.83 ± 56.12ng×hr/ml whereas in animals treated with *Ficusreligosa*L. extract was 1068.46 ± 96.4 ng×hr/ml. This higher $AUC_{0-24}$ in nanoparticles form is due to the avoidance of first pass hepatic metabolism by lymphatic transport which is one of the greatest advantages of SLN. Further, $AUC_{0-24}$ of lupeol in ESLN is 21-fold higher than plain lupeol and 8-fold higher than lupeol loaded SLN (LSLN). The improvement in higher $AUC_{0-24}$ in ESLN

than LSLN is due to the presence of polyphenols or saponins in *Ficusreligosa*L. extract which do not possess any pharmacological effect but often improve the solubility of lupeol, a marker compound of *Ficusreligiosa*L. extract and thus results in the improved bioavailability of lupeol in extract form and specially in nano-form than the marker compound's bioavailability. This result is in agreement with the literature (Wagner). In the same way, peak plasma concentration ($C_{max}$) of lupeol in ESLN was 3.5-fold higher than extract, 6.5-fold higher than plain lupeol and 1.9-fold than LSLN. The order of improved $C_{max}$in different forms is ESLN>LSLN>extract>lupeol. Time to reach plasma concentration ($t_{max}$) of lupeol in both ESLN and LSLN was 2 hours however for extract and lupeol was found to be 6 and 8 hours, respectively. $t_{1/2}$ of lupeol was found to be 7.3 ± 1.0 hours in extract and 15.3 ± 1.3 hours in ESLN, 13.8 hours in LSLN and 4.6 hours in lupeol. This can be correlated to the sustained release of drug from SLN. From these results, it clearly suggested that the pharmacokinetic profiles of lupeol have been improved in extract form of SLN than *Ficusreligiosa*Linn. extract, lupeol or lupeol loaded SLN after oral administration.

**Table 8.2. Pharmacokinetic profile of lupeol in different forms**

| Parameter | Extract | ESLN | Lupeol | LSLN |
|---|---|---|---|---|
| AUC0-24 (ng × hr/ml) | 1068.46 ± 296.4 | 9829.83 ± 456.12$^{a,b,c}$ | 452.37 ± 114.78$^{a}$ | 1184 ± 256.80$^{b}$ |
| $C_{max}$(ng/mL) | 198.65 ± 58.3 | 696.2 ± 37.9$^{a,b,c}$ | 107.30$^{a}$ | 351.00$^{b}$ |
| $T_{max}$(hr) | 6 | 2$^{a,b}$ | 8 | 2$^{b}$ |
| $t_{1/2}$ (h) | 7.3 ± 1.0 | 19.3 ± 1.3$^{a,b,c}$ | 4.6 ± 1.0 | 13.8 ± 1.6$^{b}$ |

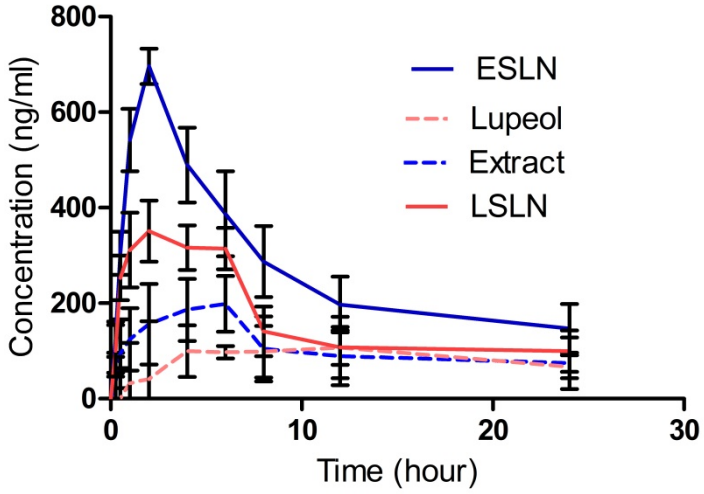


**Figure 8.13.: Plasma concentration vs. time curve of lupeol in different forms, plain lupeol, plain extract and their nano-forms; LSLN and ESLN**

Results are expressed as mean ± SEM (n=6) [a]P< 0.05compared to extract; [b]P< 0.05 compared to lupeol; [c]P< 0.05 compared to LTNPs (one-way ANOVA followed by Tukey's multiple comparison test).

## 8.4. Summary

Both ETNPs and LTNPs were prepared and they were successfully targeted into mitochondria as assessed by confocal microscopy because of functionalization using triphenylphosphonium, a mitochondrial targeting moiety whereas the untargeted EUNPs or LUNPs were failed to target mitochondria. Further, ETNPs improved mitochondrial function in oxidative stress of diabetes than EUNPs or extract or LTNPs or LUNPs or lupeol. In addition to the improvement in mitochondrial function by ETNPs treatment, they had pronounced antidiabetic efficiency than EUNPs or extract or LTNPs or LUNPs or lupeol.From the results, it is concluded that ETNPs can be used as potential alternative

to the market formulation of synthetic drugs which often result in side effect for the chronic management of diabetes.