6.1 Introduction:

Oxidative stress plays an important role in developing insulin resistance and leads to glucose intolerance[1]. The occurrence of oxidative stress is a result of overproduction of reactive oxygen species (ROS) in the cells. It has been reported that oversupply of calorie, e.g., glucose and fatty acid, generates more substrates that entered into the mitochondrial respiration process. As a result, the number of electrons donated to the electron transport chain (ETC) becomes higher. Once the membrane potential reaches its threshold limit, the electrons begin to back up at complex III and produce higher levels of superoxide radicals[2]. The oxidative stress in diabetes mellitus (DM) induces several adverse effects in cellular physiology and alters the cellular antioxidant levels. Increased oxidative damage and the reduction of the antioxidant capacity could be associated with the complications in the patients with type 2 diabetes mellitus (T2DM). It has been observed that the plasma antioxidant level is significantly lower in the patients suffering from insulin resistance and diabetes[3,4]. Superoxide dismutase (SOD), catalase, and glutathione peroxidase are the three primary enzymes which act as endogenous antioxidants, involved in direct elimination of the active oxygen species (hydroxyl radical, superoxide radical, hydrogen peroxide); whereas glutathione reductase, glucose-6-phosphate dehydrogenase, and cytosolic glutathione are secondary enzymes, which help in the detoxification of ROS by decreasing peroxide levels or maintaining a steady supply of metabolic intermediates like glutathione and NADPH which are necessary for optimum functioning of the primary antioxidant enzymes. Excess of ROS or a decrease in the antioxidant level causes tissue damage by physical, chemical, psychological factors that lead to tissue injury[5].

Recent research has shown that the antioxidants of plant origin with free-radical scavenging properties may have great importance as therapeutic agents in several diseases caused by oxidative stress[6]. Plant extracts and phytoconstituents are found effective as radical scavengers and inhibitors of lipid peroxidation. In the present study, the role of FFA on the activity of cellular antioxidant enzymes and the expression of their respective genes, consequently, targeting oxidative stress was studied as this could be a viable option to suppress lipid induced insulin resistance in near future. The effects of ethanolic extracts of *L. aspera* (LAE) and

ferulic acid (FRL) on the lipid induced insulin resistance through the upregulation of antioxidant enzymes expression and activity are also reported in this study.

6.2 Materials and Methods

6.2.1 Chemicals and reagents

All the cell culture materials were obtained from Gibco-BRL, Life Technologies Inc., Gaithersburg, MD 20884-9980, USA. 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), tricholoroacetic acid (TCA), reduced glutathione (GSH), reduced nicotinamide adenine dinucleotide (NADH), and sodium pyruvate were obtained from Sigma Chemical Co. (St Louis, MO, USA). N -(1-Naphthyl) ethylenediaminedihydrochloride (NEDD), 1-chloro-2,4-dinitrobenzene (CDNB), oxidized GSH (GSSG), pyrogallol, potassium ferricyanide, triton X-100, ethylenediaminetetraacetic acid (EDTA), sodium pyruvate, thiobarbituric acid (TBA), reduced nicotinamide adenine dinucleotide (NADH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from HiMedia Laboratories (Mumbai, India). Bovine serum albumin (BSA) was procured from Merck, India. The rest of the chemicals were of analytical grade, purchased from Sigma Aldrich, St. Louis, MO, USA, Himedia, Mumbai, India, Thermo Fisher Scientific, Waltham, MA, USA and BioBharati Life Science, India.

6.2.2 Cell culture and treatments

L6 skeletal muscle cells were maintained as described earlier. The media was changed in every 2 days and myotube differentiation was completed after 6 days of culture. Myotubes were treated without (control) or with different concentrations of SFA or LAE or both for 12 or 24 hrs. Different concentrations of FFA was prepared by conjugation of palmitate with bovine serum albumin (BSA) as described by Dey et al.[7] For treatments, the LAE was preincubated for 1 hr followed by 12 or 24 hrs incubation with FFA. On termination of incubation, the cells were washed with DPBS and harvested with trypsin-EDTA solution. Cell pellets were resuspended in lysis buffer (Invitrogen). Lysates were centrifuged at 10000 rpm for 10 mins at 4ºC and protein concentrations were determined by following the method of Lowry, et al.[8]

6.2.3 Measurement of lipid peroxidation (LP)

Peroxidation of lipid was estimated spectrophotometrically by the assay of thiobarbituric acid reactive substances (TBARS) and expressed in terms of nM of malondialdehyde (MDA) formed per mg protein[9]. Briefly, in a 3 ml reaction volume cell homogenate was mixed with 0.15 M Tris–KCl buffer (pH 7.4) and 30 % tricholoroacetic acid (TCA) and 52 mM thiobarbituric acid (TBA). The mixture was heated for 45 minutes at 80ºC, cooled and centrifuged for 10 minutes at 3000 rpm. The absorbance of the supernatant was measured at 531.8 nm in spectrophotometer (Thermo Scientific, UV 10).

6.2.4 Measurement of reduced glutathione (GSH)

Reduced glutathione level was measured following the procedure described by Moron et al., 1979[10]. In brief, the cell lysate was treated with tricholoroacetic acid (TCA) to precipitate cellular protein, and supernatant was collected. The collected supernatant was mixed with 0.6 M 5, 5'-dithiobis 2-nitrobenzoic acid and allowed to stand for 8–10 min at room temperature. The absorbance was recorded at 412 nm using a spectrophotometer (Multiskan go, Thermo Scientific, Finland). Reduced glutathione (GSH) was used as a standard to calculate nM of –SH content/mg protein and finally expressed as percentage change of GSH levels as compared to the control cells.

6.2.5 Measurement of Nitric oxide (NO)

Nitric oxide (NO) was measured as a nitrite level in the cell lysate by the method by Griess, 1879[11]. The reaction mixture (200 µl) containing equal volume of cell lysate and Griess reagent (prepared by mixing equal volume of 0.2% NEDD in 95% ethanol and 2% sulfanilamide in 10% orthophosphoric acid) was incubated at dark for 30 minutes. The absorbance was measured at 550 nm in a microplate reader (Multiskan Ascent, Thermo Electron Corporation). Sodium nitrite was used as a standard to calculate in mM NO/mg protein and finally expressed as percentage change of NO level as compared to the control cells.

6.2.6 Superoxide dismutase (SOD) activity

SOD activity was analysed by the method of Marklund and Marklund, 1974[12]. In brief, the cell lysate was mixed with assay buffer containing 0.1 mM EDTA and pyrogallol in 50 mM phosphate buffer (pH 8.0) and absorbance was measured for 2 mins at 420 nm. A single unit of enzyme is defined as the quantity of SOD required to produce 50% inhibition of autoxidation.

6.2.7 Catalase (CAT) activity

Catalase activity was measured monitoring the decomposition of hydrogen peroxide (H_2O_2), as described by Kawamura, N., 1999[13]. In brief, the cell lysate was mixed with assay buffer containing 10 mM H_2O_2 in100 mM sodium phosphate buffer (pH 7.0) and absorbance was recorded at 240 nm in spectrophotometer for 3 mins. The specific activity of catalase has been expressed as moles of H_2O_2 reduced $min^{-1}mg^{-1}$ protein.

6.2.8 Glutathione peroxidise (GPx) activity

GSH peroxidase activity (GPX) was measured by the coupled assay method as described by Paglia and Valentine, 1967[14]. Briefly, the cell lysates were incubated for 1 min with 0.1 M Na-K phosphate buffer (pH 7.0), 1 mM EDTA and 30 mM reduced glutathione followed by incubation for 0.5 min by adding 2 mM H_2O_2. The mixture was allowed to react with glutathione reductase and 2 mM NADPH, and the absorbance was measured at 340 nm. The decrease in absorbance was observed for 3 min. One unit of enzyme activity has been defined as nM of NADPH consumed/min/mg protein based on an extinction coefficient of 6.22 $mM^{-1}cm^{-1}$.

6.2.9 Glutathione reductase (GR) activity

Glutathione reductase activity was determined by the method described earlier by Carlberg and Mannervik, 1985[15]. The reaction was initiated by adding the sample in milli-Q water containing 2 mM EDTA, 20 mM GSSG and NADPH. Further, the absorbance was recorded at 340 nm wavelength for 3 mins. One unit of enzyme activity is expressed as nM of NADPH consumed/min/mg protein, where the extinction coefficient of GR used is 6.22 $mM^{-1}cm^{-1}$.

6.2.10 RNA isolation and cDNA synthesis

Total RNA was extracted from the control and the treated L6 myotubes using TRI reagent (Sigma- Aldrich) and reverse transcription was performed using Super Reverse Transcriptase MulV Kit (BioBharati Life Science, India) according to the manufacturer protocol.

6.2.11 Semi quantitative RT-PCR

Semiquantitative PCR was performed to check the relative expression of the mRNA of the target genes. The amplification of the cDNA of the respective mRNA was performed using *Taq* DNA Polymerase plus kit (BioBharati Life Science, Kolkata, India) with a little modification of the manufacturer protocol.

6.2.12 Statistical analysis

All data were derived from at least three independent experiments and statistical analyses were conducted using Microsoft Excel. Data were analysed by one way analysis of variance (ANOVA), where the p value indicated significant differences between group means. All values were means ± SEM. A level of $p < 0.05$ was considered as significant.

6.3 Results

6.3.1 Saturated free fatty acid alters the levels of LP, GSH and NO in treated cells

Lipid peroxidation is a key event in oxidative stress resulting in malondialdehyde (MDA) and conjugated diene formation. Hence the formation of MDA and conjugated diene is more in case of cells having oxidative stress[16], while the level of GSH is less during oxidative stress. Since GSH helps in detoxification of free radicals from the cells, depletion of GSH level inside the cells increases the risk of developing oxidative stress resulting in insulin resistance and T2DM. Similarly, nitric oxide (NO), a key regulatory molecule, plays a vital role in developing oxidative stress. Though NO is beneficial for both physiological and cellular function at a lower level, higher levels are harmful to the cell as it generates peroxynitrile radicals and affects the function of cellular proteins by

binding to them[17]. Since free fatty acid is one of the key players of insulin resistance and also for developing oxidative stress, it was necessary to investigate as to how FFA alters the rate of lipid peroxidation along with the alteration of GSH and NO levels. To address as to how FFA influences muscle cells to develop oxidative stress, L6 cells were treated with various concentrations of palmitate for different time periods. Altered levels of lipid peroxidation, GSH and NO were checked in both normal and the treated cells. It was found that FFA induced the level of MDA formation in both time and concentration dependent manner implying the induction of lipid peroxidation (Fig. 6.1A). The level of GSH was also found to be depleted in the cells that were treated with FFA. From Fig 6.1B, it was observed that the depletion of GSH level was also dependent on FFA concentration and time of incubation. However, the level of NO was found to be increased with increasing concentration of FFA at 12 and 24 hrs of incubation (Fig. 6.1C)

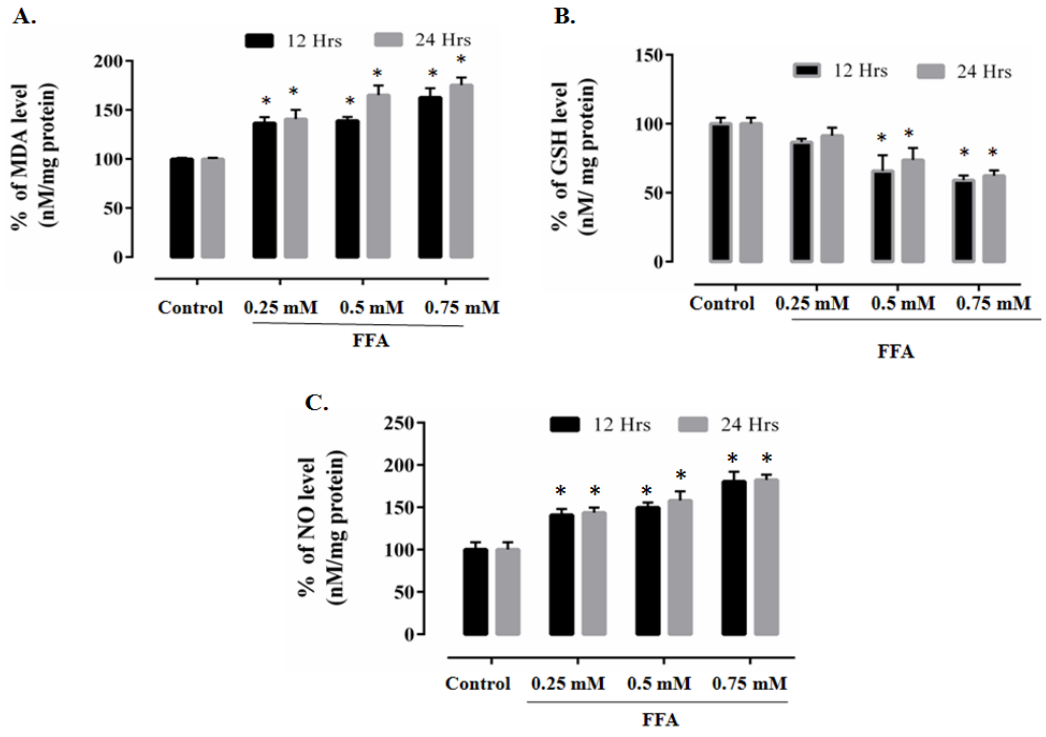

Figure 6.1: Effect of FFA in alteration of biological oxidative markers. Alteration of Lipid peroxidation (A), GSH level (B) and NO level (C) in L6 cells treated with or without palmitate or palmitate for 12 and 24 hrs. Each value is the mean±SEM of three independent experiments, *p<0.05 vs Control.

6.3.2 Alteration of antioxidant enzyme genes expression and their activity by the action of saturated fatty acid

In normal cellular mechanism, the elevated level of ROS molecules can be neutralized both enzymatically and non-enzymatically by the antioxidant system of living system[18]. In the enzymatic defence mechanism, several enzymes, e.g., superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidise (GPx) acts as the primary enzymes for protecting the cell from pro-oxidants. Secondary antioxidant enzymes also play a vital role in reducing the oxidative stress in the cells[19,20]. Since it has been observed that the elevated level of FFA induces the level of oxidative stress in the treated L6 muscle cells, it was studied whether the elevated levels of FFA has any effect on expression and activities of primary antioxidant enzymes. Cells were treated with different concentrations of palmitate for 2 different time periods and it was found that treatment with palmitate reduced the activity of SOD in both concentrations and in a time dependent manner (Fig. 6.2A). The expression pattern of the SOD gene was also observed at similar conditions and it was found that when FFA was treated with increasing concentrations, the expression became less gradually (Fig. 6.2B)

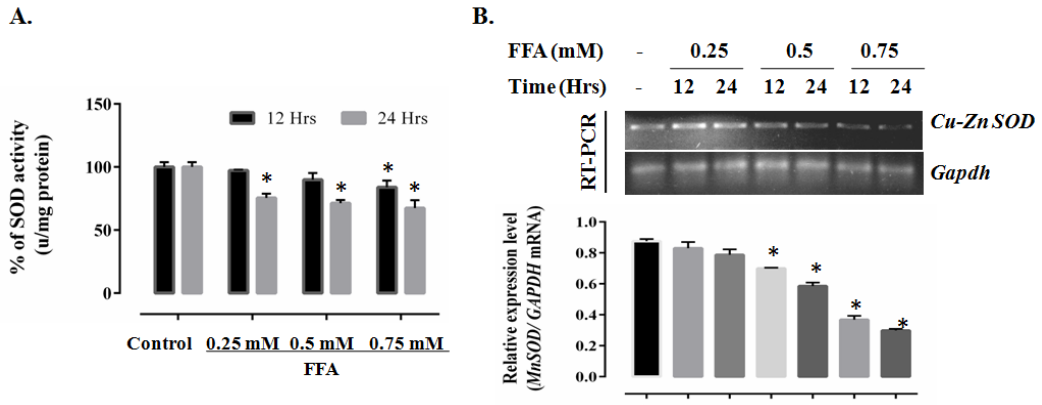

Figure 6.2: FFA reduced the gene expression and enzymatic activity of superoxide dismutase (SOD) in muscle cells. (A) Effect of different concentrations of FFA in SOD activity in L6 skeletal muscle cells incubated with FFA for 12 and 24 hrs; (B) RT-PCR analysis showing the relative expression of Cu-Zn SOD mRNA in L6 skeletal muscle cells treated with various concentrations of FFA for 12 and 24 hrs. Each value is the mean±SEM of three independent experiments, *p<0.05 vs Control.

The same experiment showed that the activity of CAT also decreased with increasing concentration of FFA (Fig.6.3A). The gene expression analysis revealed

the downregulation of CAT gene expression with increasing concentrations of FFA (Fig.6.3B).

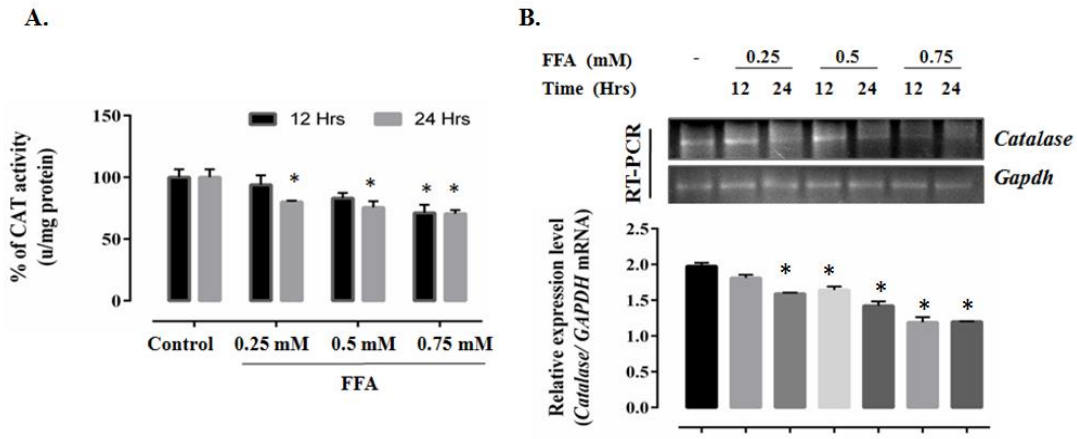

Figure 6.3: FFA reduced the gene expression and enzymatic activity of Catalase (CAT) in muscle cells. (A) Effect of different concentrations of FFA in CAT activity in L6 skeletal muscle cells incubated with FFA for 12 and 24 hrs; (B) RT-PCR analysis showing the relative expression of CAT mRNA in L6 skeletal muscle cells treated with various concentrations of FFA for 12 and 24 hrs. Each value is the mean±SEM of three independent experiments, *p<0.05 vs Control.

GPx is the enzyme which plays an important role in converting H_2O_2 into water molecule in the cytoplasm[21]. So to maintain the normal redox state in the body, the activity of GPx should be balanced. The GPx activity was investigated in the FFA treated L6 muscle cells to check its effect on the GPx activity. From the result obtained, it was found that the activity of GPx gradually decreased with the increase in the concentrations of FFA and the time of incubation (Fig. 6.4A). Since GPx-1 is the common isoform of GPx, its expression profile after the cells treated with FFA was studied. The gene expression profile of GPx -1 reveals that like the other two primary antioxidant genes expression, FFA treatment also reduced the expression of GPx-1 in the FFA treated L6 muscle cells (Fig. 6.4B).

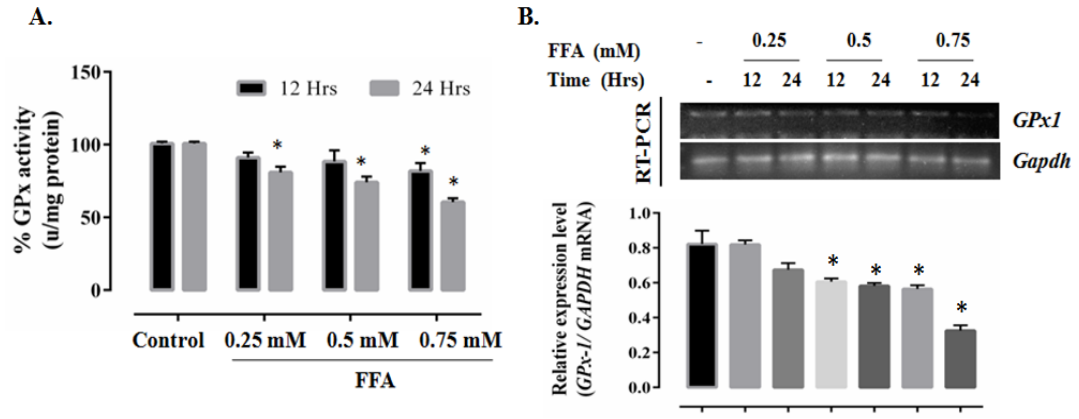

Figure 6.4: FFA reduced the gene expression and enzymatic activity of glutathione peroxidise (GPx) in muscle cells. (A) Effect of different concentrations of FFA in GPx activity in L6 skeletal muscle cells incubated with FFA for 12 and 24 hrs; (B) RT-PCR analysis showing the relative expression of GPx-1 mRNA in L6 skeletal muscle cells treated with various concentrations of FFA for 12 and 24 hrs. Each value is the mean±SEM of three independent experiments, *p<0.05 vs Control.

Glutathione reductase (GR) is also a central antioxidant enzyme in the cellular antioxidant defence system. It plays its role in reducing the oxidised form of glutathione (GSSG) to the sulfhydril form GSH[22]. Several reports are available suggesting the decreased activity of GR in the patients suffering from diabetes[23]. The activity of GR in L6 skeletal muscle cells in presence of various concentrations of FFA in two different time periods was studied. The results obtained from the study showed that when the cells were treated with increasing concentration of FFA for 12 hrs, the activity of GR decreased. The decrease in of GR activity was found to be more in the case of the cells treated with FFA for 24 hrs (Fig 6.5), suggesting that FFA reduces GR activity in both concentrations and in a time dependent manner in the muscle cells.

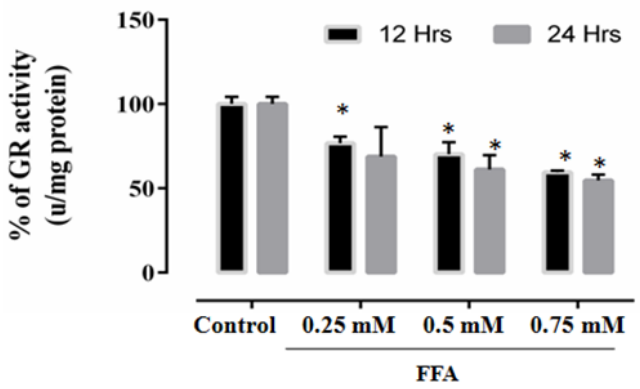

Figure 6.5: FFA reduced the enzymatic activity of glutathione reductase (GR) in muscle cells. Enzyme activity study showing the effect of different concentrations of FFA in GPx activity in muscle cells treated with FFA for 12 and 24 hrs;

6.3.3 Improvement of SFA alters GSH, NO, LP and LDH levels by the *L. aspera* ethanol extract and ferulic acid (FRL)

Natural products are known to be rich in antioxidants due to the presence of numerous numbers of polyphenols[24]. To study whether the LAE and FRL can reduce the FFA induced oxidative stress in L6 cells, the cells were pretreated with different concentrations of the LAE and 10 μg/ml of FRL for 24 hrs. From the result it was observed that LAE could suppress the FFA induced MDA formation in presence of FFA which was found more in FFA treated cells (Fig. 6.6A). From the Fig 6.6C it was also observed that upon pre treatment with the LAE in FFA treated L6 muscle cells, the level of GSH increased with increasing concentrations of the LAE. On the contrary, the level of NO decreased gradually with the increasing LAE concentration (Fig. 6.6E). Similarly, the effect of FRL in amelioration of FFA induced alterations of lipid peroxidation was studied along with the levels of GSH and NO in the L6 muscle cells. It has been found that in the cells pretreated with FFA, FRL reduced the formation of MDA and the level of NO (Fig. 6.6B and 6.6F). FRL markedly increased the GSH level (almost 1.5 folds) in the cells treated with FFA for 24 hrs (Fig. 6.6D).

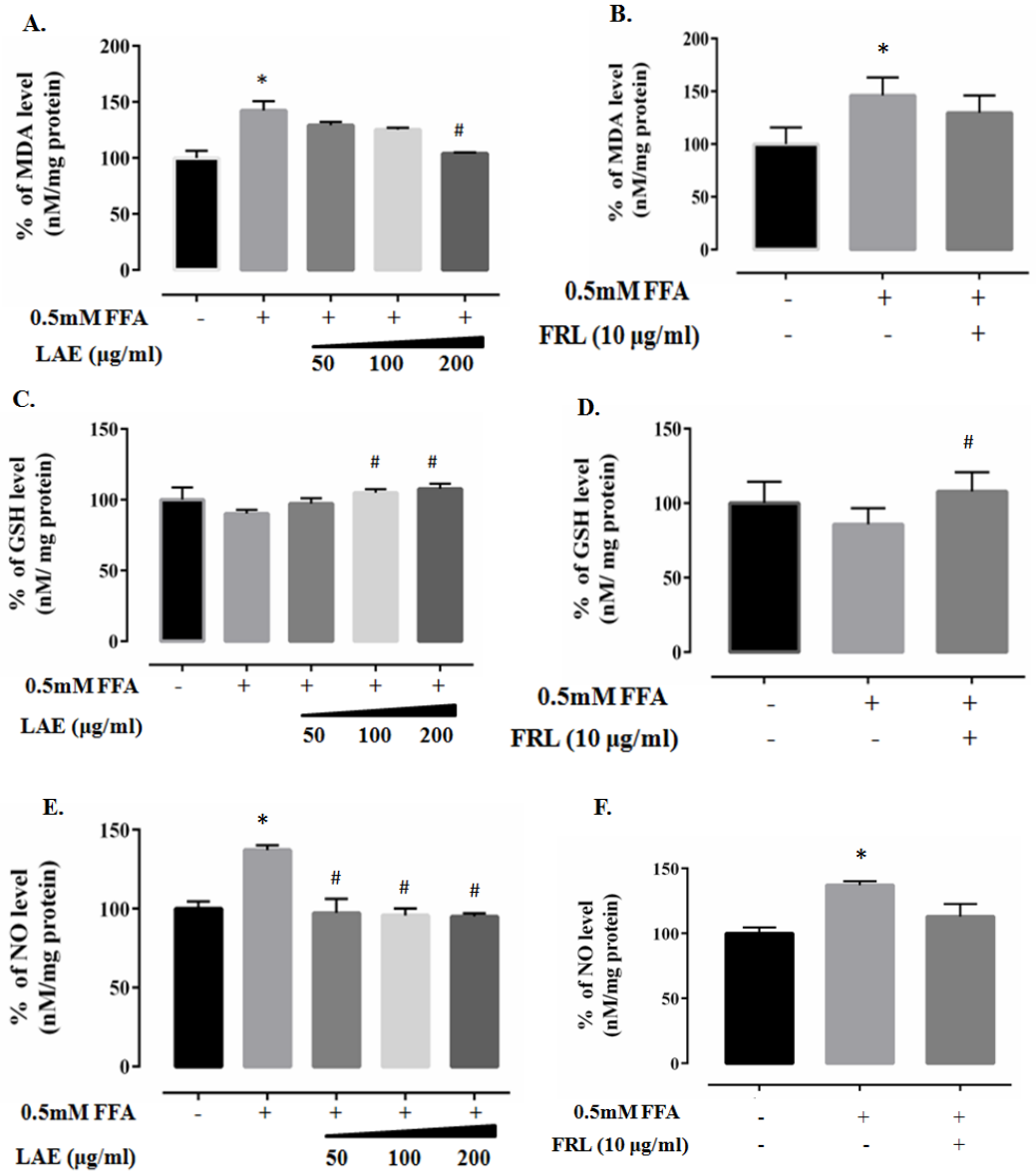

Figure 6.6: Effect of LAE and FRL in FFA induced alteration of biological oxidative markers. LAE reduced the (A) lipid oxidation and (E) NO production induced by FFA whereas (C) reduced FFA suppressed GSH level in L6 skeletal muscle cells. Similarly, FRL suppressed FFA induced (B) lipid peroxidation and (F) NO production whereas (D) it helps in suppressing FFA activity in reducing the GSH level in L6 skeletal muscle cells. Each value is the mean±SEM of three independent experiments, *$p<0.05$ vs Control, $^{\#}p<0.05$ vs FFA.

6.3.4 Modulation of SFA altered antioxidant enzyme genes expression and their activity by LAE and FRL.

Increased level of ROS induces the risk of oxidative stress which poses a threat to the health of both humans and animals resulting in the dysfunction of various cell types in the body[25]. Natural products are known to be potent antioxidants which neutralize the ROS generated in the cell in two ways. It either neutralizes the ROS generated in the cell due to its inherent antioxidant property, or it modulates the antioxidant enzymes activity. To check the effect of the LAE and FRL in FFA altered antioxidant enzymes activity as well as in their respective gene expression in the FFA treated L6 cells, the cells were pretreated with different concentrations of the LAE or 10μg/ml concentration of FRL for 24 hrs. From the result obtained, it was found that pretreatment of L6 muscle cells with LAE, FFA could not reduce CAT activity (Fig. 6.7A). To confirm if the changes occurred due to change in molecular level or not, RT PCR analysis was performed. RT-PCR analysis revealed that by the treatment of different concentrations of the LAE, the expression of the CAT gene was increased with increasing concentrations of LAE in presence of FFA which was found to be depleted in only the FFA treated cells (Fig. 6.7B). Similar pattern of the results were also obtained in the FRL treated cells where increased CAT gene expression was obvious. This activity was earlier reduced by FFA and on treatment with FRL, it can be seen that there has been an increase of the activity at enzymatic level (Fig. 6.7C). Induced level of CAT gene expression was also found in FFA treated cells pretreated with FRL (Fig. 6.7D)

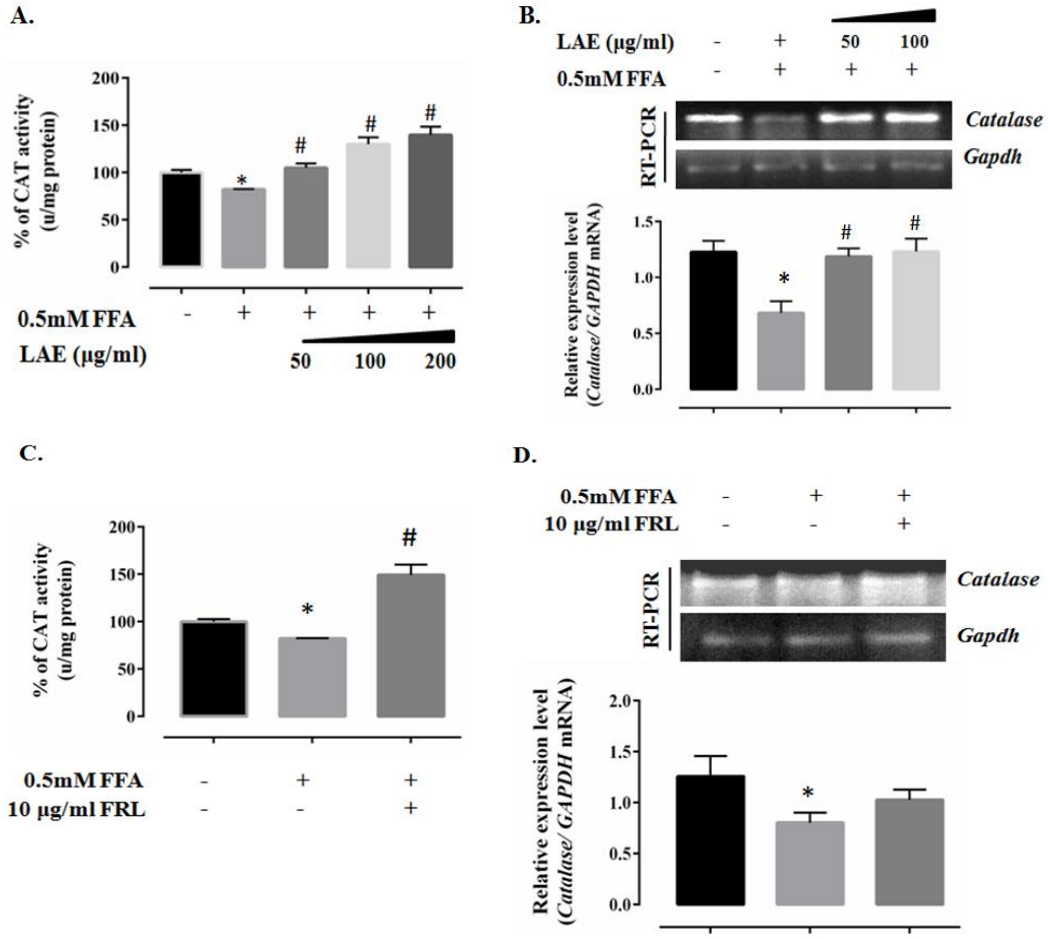

Figure 6.7: Effect of LAE and FRL in increasing catalase (CAT) activity and CAT mRNA expression in FFA treated cells. Pretreatment of FFA treated L6 cells with LAE suppresed the effect FFA in reducing the (A) CAT activity and (B) its expression. Similarly, cells pretreated with FRL also reduced the activity of FFA in reducing (C) CAT activity and (D) expression. Each value is the mean±SEM of three independent experiments, *$p<0.05$ vs Control, $^{\#}p<0.05$ vs FFA.

The effect of the LAE and FRL in SOD activity on the FFA treated L6 cells were also analysed. From the Fig 6.8A it can be seen that SOD activity was also increased in the FFA treated L6 cells with increasing concentrations of LAE in the same way as it was observed in the case of CAT activity. Increased activity of SOD was also observed in the cells that were subjected to pretreatment with FRL (10µg/ml) in presence of FFA (Fig. 6.8C). RT-PCR analysis revealed that, by the treatment of the LAE and FRL, the loss of SOD expression by FFA can be reverted by LAE extract or FRL in L6 muscle cells in presence of FFA (Fig. 6.8 B and D).

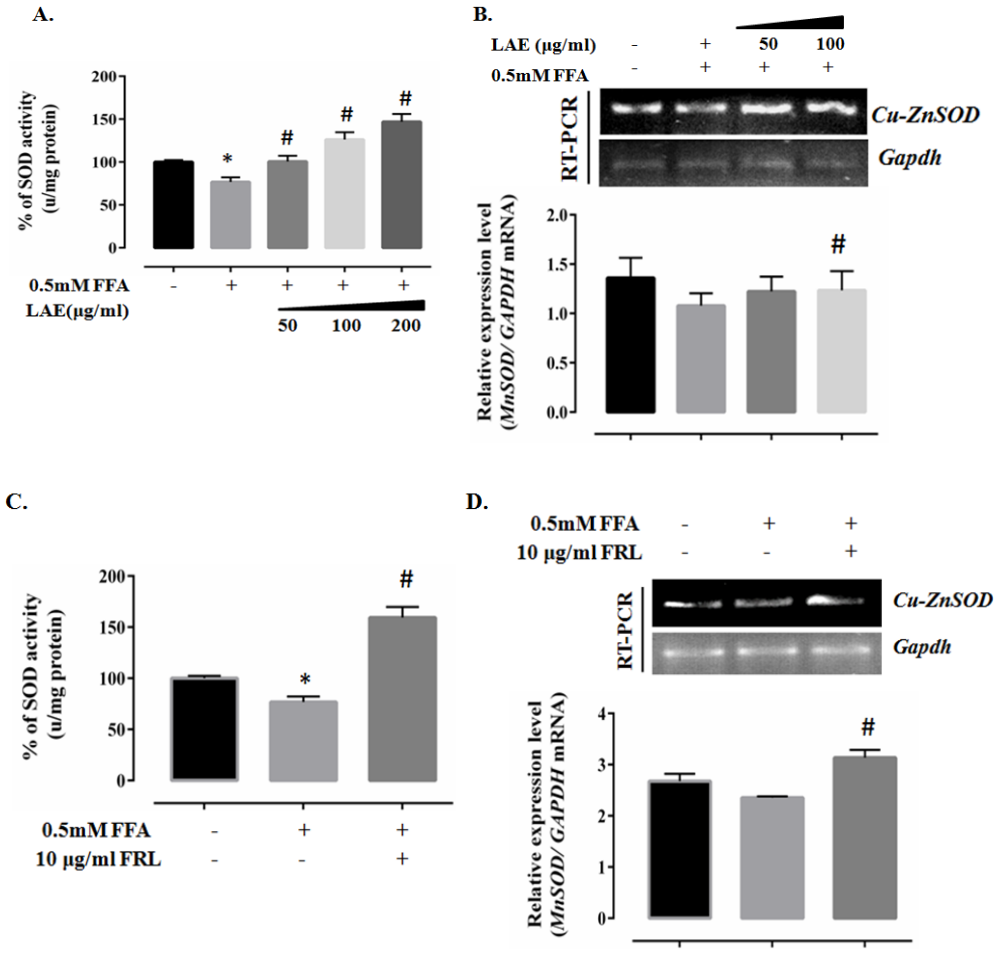

Figure 6.8: Effect of LAE and FRL in increasing superoxide dismutase (SOD) activity and SOD mRNA expression in the FFA treated cells. FFA pretreated L6 cells with the LAE suppresed the effect FFA in reducing the (A) SOD activity and (B) its expression. Similarly, cells pretreated with FRL also reduced the activity of FFA in reducing (C) SOD activity and (D) expression. Each value is the mean±SEM of three independent experiments, *p<0.05 vs Control, #p<0.05 vs FFA.

Along with CAT and SOD, the effect of LEA and FRL were analysed on the activity and gene expression of GPx. The activity of GPx decreased under the influence of FFA while it was increased when the cells were treated with different concentrations of the LAE (Fig. 6.9A). RT-PCR analysis showed the downregulation of the GPx-1 gene expression in presence of FFA whereas it was found to be upregulated by the treatment of the L6 cells with the LAE (Fig. 6.9B). These results suggest that the LAE can induce the GPx gene expression in concentration dependent manner effecting in its activity. Treatment with 10µg/ml FRL also generated similar trends of inference (Fig. 6.9 C and D).

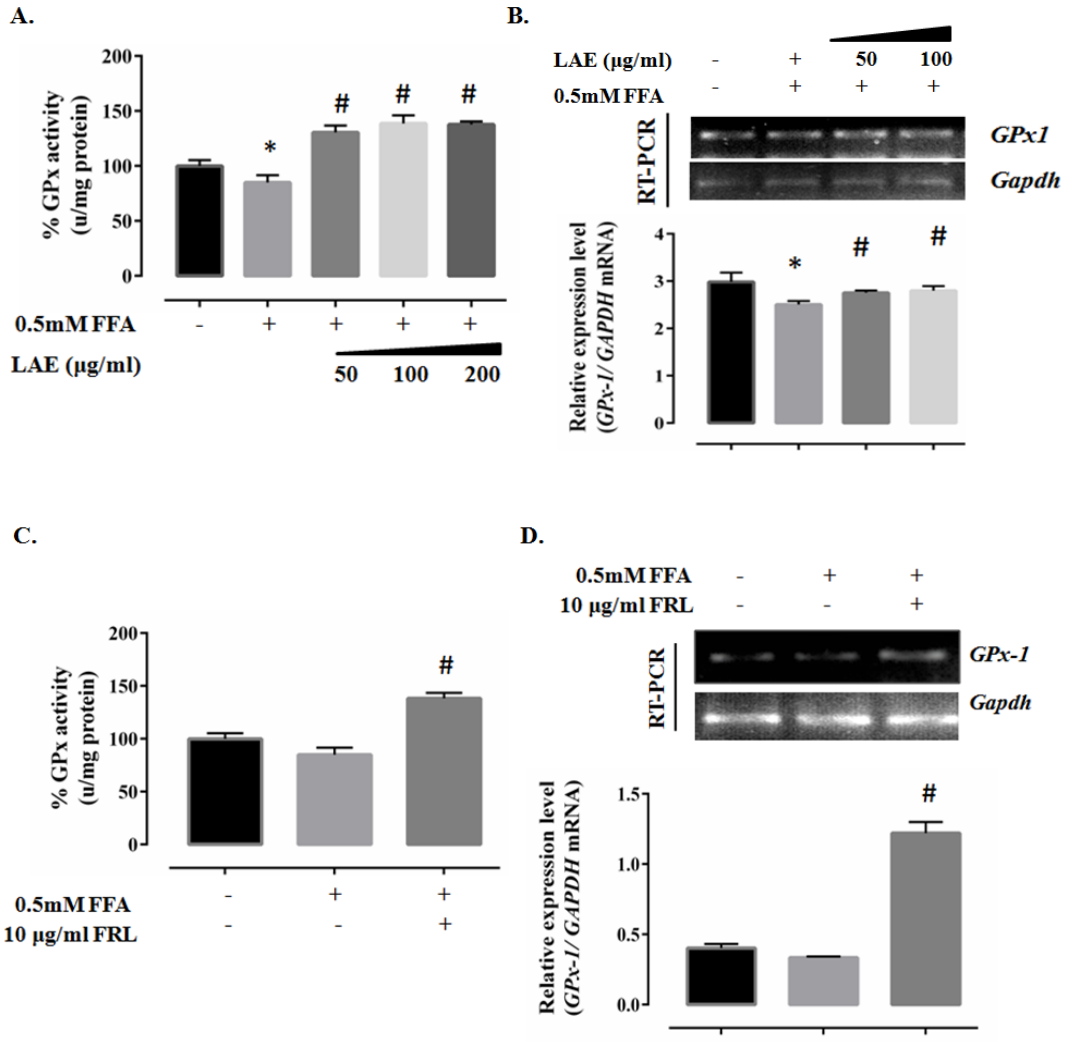

Figure 6.9: LAE and FRL increased the glutathione peroxidase (GPx) activity and GPx mRNA expression in FFA treated cells.L6 cells pretreated with FFA showed the effect of FFA in reducing the (A) GPx activity and (B) in expression of GPx-1 gene, on being treated with LAE. Similarly, cells pretreated with FRL also reduced the (C) activity of FFA in reducing GPx activity and (D) GPx-1 gene expression. Each value is the mean±SEM of three independent experiments, *$p<0.05$ vs Control, $^{\#}p<0.05$ vs FFA.

Since FFA results in the depletion of the GR activity in a concentration and time dependent manner, the activity of GR was investigated by the treatment of the LAE and FRL in presence of FFA. The LAE and FRL are known to have antioxidant properties. This study also showed that the GR activity was increased significantly with the increase in the concentrations of the LAE and FRL (Fig. 6.10 A and B).

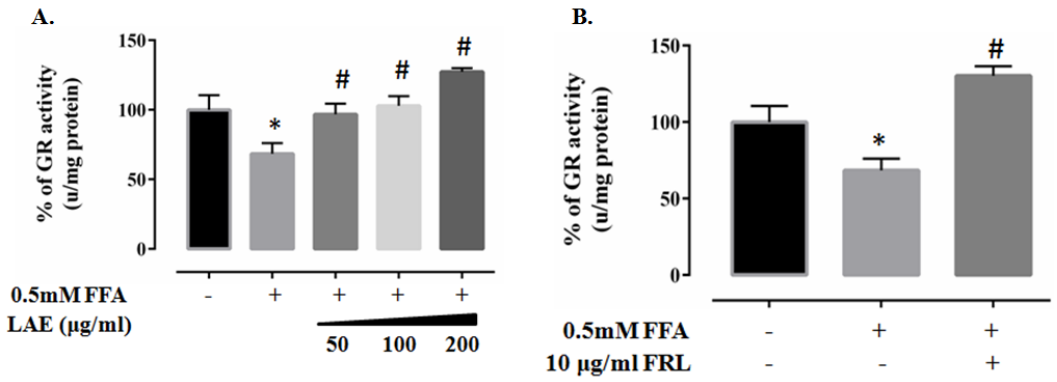

Figure 6.10: LAE and FRL increased the glutathione reductase (GR) activity in the FFA treated cells. Enzyme activity study showing the effect of different concentrations of (A) LAE and (B) 10µg/ml FRL in GR activity in presence of FFA. Each value is the mean±SEM of three independent experiments, *p<0.05 vs Control, #p<0.05 vs FFA.

6.4 Discussion

Saturated free fatty acids (FFAs) are one of the most crucial links between obesity and insulin resistance and T2DM. Insulin stimulates postprandial absorption and storage of glucose and the skeletal muscle acts as the primary site for carbohydrate deposition. Simultaneous exposure of muscle fibres to FFA or lipoprotein-bound triglycerides eventually leads to the development of insulin resistance, a metabolic state which predisposes subjects to cardiovascular disease and T2DM[26]. Several reports suggest that insulin resistance and diabetes are associated with the alteration of cellular redox balance imposed by increasing concentration of lipid[27,28].It was reported that increased accumulation of lipid causes the generation of ROS which ultimately leads to the insulin resistant state of the cell[28]. The excessive lipid accumulation in the cell induces mitochondrial dysfunction leading to ROS production which in turn causes mitochondrial dysfunction in a cyclic manner[29,30]. The large amount of ROS production in this way causes oxidative stress inside the cell. The primary molecules that play the protective roles in ROS management inside the cells are the antioxidant enzymes. Though in presence of the cellular antioxidant system, ROS develops oxidative stress in the cell, the antioxidant activity of these enzymes may not be sufficient to neutralise the effect of ROS. Several reports suggest that a significant downregulation of SOD expression was observed in the obese diabetic patients[31]. Patients suffering from diabetes were also reported to be deficient in catalase

activity which leads to increased abundance of H_2O_2 in the cellular environment. This in turn induces oxidative stress making the individual more prone to insulin resistance[32]. In addition to this, the reduction of total antioxidant activity, including GPx activity was also evident in T2DM subjects. Since, FFA is intimately associated with obesity and consequently with T2DM, therefore, it is pertinent to investigate the effect of FFA if any, on the cellular antioxidant system. As such, L6 skeletal muscle cells were treated with different concentrations of palmitate, a saturated free fatty acid (FFA) for 2 different time periods. Since 80-90% of post prandial glucose disposal occurs in skeletal muscle cells, rat skeletal L6 muscle cells were used in this study. In addition, it has also been established that palmitate incubation strongly induces oxidative stress in the skeletal muscle cells[33]. Since FFA is a prime factor of insulin resistance, the effect of FFA in pro-oxidant level was investigated. FFA treatment of L6 myotubes increased the lipid peroxidation as indicated by enhancement of MDA formation along with the lower content of GSH in the FFA treated cells (Fig. 6.1 A and B). This further suggested the development of oxidative stress in the cells by the treatment of FFA. The higher level of NO production in FFA treated L6 skeletal muscle cells confirmed the development of oxidative stress because palmitate induces NO production *via.* the nuclear factor κB (NFκB) activation and increases production of superoxide and iNOS protein content in the skeletal muscle cells[34]. In the present study, the impact of FFA on the antioxidant gene expression and activity was examined which revealed antagonistic effects on the gene expression and activity, as the concentration of FFA was inversely proportional to their expression and activity (Fig. 6.2, 6.3, 6.4). Therefore, it can be hypothesized that FFA induces oxidative stress in the cultured L6 skeletal muscle cells in two ways, i.e., i) by increasing the ROS production through mitochondrial dysfunction and ii) by reducing the expression of antioxidant genes which lowers the activity of cellular antioxidant enzymes. As a result, cells are not able to produce sufficient amount of antioxidant enzymes to neutralise the ROS produced due to mitochondrial dysfunction. This induces oxidative stress in L6 skeletal muscle cells resulting into insulin resistance. Therefore, oxidative stress could be a good therapeutic target for suppression of lipid induced insulin resistance.

In the present work, the effect of LAE extract and FRL on insulin sensitivity in L6 skeletal muscle cells was examined. The study revealed that LAE extract and FRL potentially elevated insulin sensitivity in FFA incubated insulin resistant cells, indicating its potential antioxidative properties. Therefore, to validate the hypothesis its effect on cellular antioxidant system was examined. Pre treatment of insulin resistant L6 cells with LAE and FRL effectively ameliorated FFA induced alteration of the levels of oxidative stress markers. Recently, in a different study Kripa, et al. 2011 reported the modulatory effect of some antioxidant markers in adjuvant arthritis by *L. aspera* extract[35]. The enzymatic activities of cellular antioxidants were also found to be increased in FFA treated L6 muscle cells by the treatment of LAE and FRL. Interestingly, gene expression study revealed that FFA induced downregulation of antioxidant genes could be improved by the treatment of LAE as well as FRL. These results suggest that the pre-treatment of L6 muscle cells with the ethanolic extract of *L. aspera* and FRL can prevent the action of FFA in the antioxidant gene regulation of the and thereby sufficient amount of antioxidant enzymes are synthesised. The synthesis of various enzymes showed more enzymatic activity to neutralise the ROS generated due to FFA induced mitochondrial dysfunction. This in turn reduces oxidative stress in the FFA treated L6 cells resulting into the reduced insulin resistance and increased glucose uptake even in presence of FFA.

References:

1. Negre-Salvayre, A., et al. Hyperglycemia and glycation in diabetic complications, *Antioxidants and Redox Signaling* **11**(12), 3071--3109, 2009.
2. Brownlee M. Biochemistry and molecular cell biology of diabetic complications, *Nature* **414**,813--820, 2001.
3. Archuleta, T.L., et al. Oxidant stress-induced loss of IRS-1 and IRS-2 proteins in rat skeletal muscle: role of p38 MAPK, *Free RadicBiol Med*. **47**, 1486--1493, 2009.
4. Kamata, H, et al. Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases, *Cell* **120,** 649--661, 2005.
5. Tian, Y., et al.Neuroprotective effect of catalpol against MPP+-induced oxidative stress in mesencephalicneurons,*European Journal of Pharmacology* **568,** 142--148, 2007.
6. Ramchoun, M., et al. Study on antioxidant and hypolipidemic effects of polyphenol rich extract from Thymus vulgaris and Lavendulamultifidi, *Pharmacognosy Research* **1,** 106--112, 2009.
7. Dey, D, et al. Inhibition of insulin receptor gene expression and insulin signaling by fatty acid: interplay of PKC isoforms therein, *Cell Physiol Biochem* **16** (4--6), 217-28, 2005.
8. Lowry, O. H., et al. Protein measurement with the folin phenol reagent, *J. Biol. Chem.***193**, 265--275, 1951.
9. Gutteridge, J.M.,& Quinlan, G.J. Malondialdehyde formation from lipid peroxides in the thiobarbituric acid test: the role of lipid radicals, iron salts, and metal chelators, *J ApplBiochem* **5**(4--5), 293-9, 1983.
10. Moron, M.S., et al. Levels of glutathione, glutathione reductase and glutathione-S-transferase activities in rat and lung liver, *Biochem. Biophys. Acta*. **582,** 3170--3185. 1979.
11. Griess, P.Bemerkungenzu der abhandlung der HH: Wesely und Benedikt 'UbereinigeAzoverbubdungen', *BerDeutchChemGes***12**, 426 -- 8, 1879.

12. Marklund, S.,& Marklund, G.Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase, *Eur J Biochem,* **47**(3), 469--74, 1974.
13. Kawamura, N. *Experimental protocols for reactive oxygen and nitrogen species,* Oxford University Press, New York, 1999.
14. Paglia, D.E.,& Valentine, W.N.Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase, *J Lab Clin Med*. **70**(1), 158--69, 1967.
15. Carlberg, I., & Mannervik, B. Glutathione Reductase,*Methods in Enzymology* **113**, 484--490, 1985.
16. Tangvarasittichai,S. Oxidative stress, insulin resistance, dyslipidemia and type 2 diabetes mellitus, *World J Diabetes* **6**(3), 456--480, 2015.
17. Adela, R., et al. Hyperglycaemia Enhances Nitric Oxide Production in Diabetes: A Study from South Indian Patients, *PloS one* **10** (4), 2015.
18. Pham-Huy, L. A, et al. Free Radicals, Antioxidants in Disease and Health,*Int J Biomed Sci.* **4**(2), 89--96, 2008.
19. Maser, R. L., et al. Oxidant Stress and Reduced Antioxidant Enzyme Protection in Polycystic Kidney Disease, *JASN* **13** (4), 991--999, 2002.
20. Rahal, A., et al.Oxidative Stress, Prooxidants, and Antioxidants: The Interplay, *BioMed Research International*, **2014**, 19, 2014.
21. Halliwell, B.&Gutteridge,J. M. C. *Free Radicals in Biology and Medicine*, Oxford, UK, 1989.
22. Morris, D., et al. Glutathione and infection, *Biochimica et Biophysica Acta*, **1830**(5), 3329--3349, 2013.
23. Murakami, K., et al. Impairment of glutathione metabolism in erythrocytes from patients with diabetes mellitus, *Metabolism,* **38**(8), 753--758, 1998.
24. Bahadoran, Z., et al. Dietary polyphenols as potential nutraceuticals in management of diabetes: a review, *Journal of diabetes and metabolic disorders* **12**(1), 1, 2013.
25. Yang, T., et al. Alteration of antioxidant enzymes and associated genes induced by grape seed extracts in the primary muscle cells of goats in vitro, *PloS one* **9** (9), 2014.

26. Holland, W.L, et al. Lipid-induced insulin resistance mediated by the proinflammatory receptor TLR4 requires saturated fatty acid–induced ceramide biosynthesis in mice, *The Journal of Clinical Investigation* **121**(5), 1858--1870, 2011.
27. Paglialunga, S., et al. Targeting of mitochondrial reactive oxygen species production does not avert lipid-induced insulin resistance in muscle tissue from mice, *Diabetologia* **55**, 2759--2768, 2012.
28. Furukawa, S., et al. Increased oxidative stress in obesity and its impact on metabolic syndrome,*J. Clin. Invest.* **114,** 1752--1761, 2004.
29. Hirabara, S.M., et al. Saturated fatty acid-induced insulin resistance is associated with mitochondrial dysfunction in skeletal muscle cells, *J Cell Physiol*. **222**(1), 187--94, 2010.
30. Murphy, M. P. Mitochondrial Dysfunction Indirectly Elevates ROS Production by the Endoplasmic Reticulum, *Cell Metabolism* **18**, 145--146, 2013.
31. Tiwari, B. K., et al. Markers of oxidative stress during diabetes mellitus, *Journal of Biomarkers* **2013**(378790), 8 pages, 2013.
32. Góth, L. Catalase Deficiency and Type 2 Diabetes, *Diabetes Care* **31**(12), 93, 2008.
33. Martins, A. R, et al. Mechanisms underlying skeletal muscle insulin resistance induced by fatty acids: importance of the mitochondrial function, *Lipids Health Dis*.**11**(30), 1--11, 2012.
34. Lambertucci, R.H., et al.The effects of palmitic acid on nitric oxide production by rat skeletal muscle: mechanism via superoxide and iNOS activation, *Cell PhysiolBiochem.* **30**(5), 1169--80, 2012.
35. Kripa, K.G., et al. Modulation of inflammatory markers by the ethanolic extract of*Leucasaspera* in adjuvant arthritis, *Journal of Ethnopharmacology* **134**(3), 1024--1027, 2011.