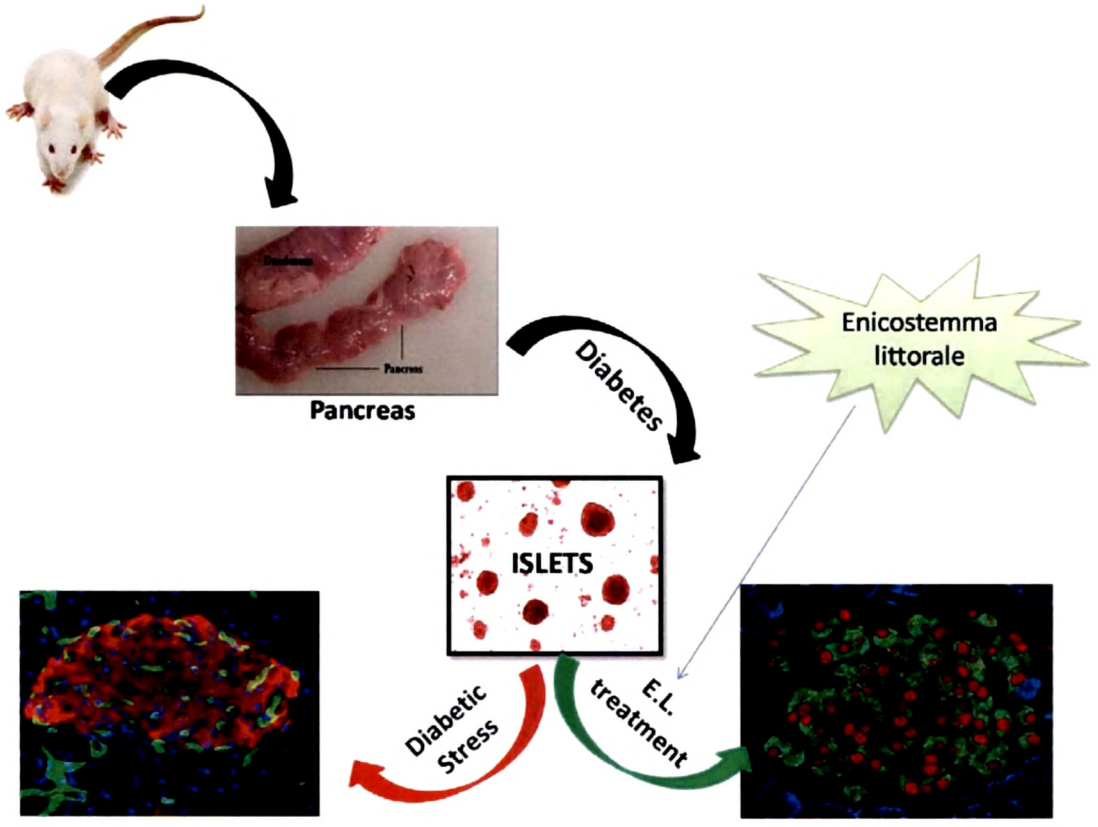
Chapter 4:

Evaluation of protective effect of *Enicostemma littorale* methanolic extract against H2O2 induced apoptosis of islets of langerhans.



Chapter 4 Evaluation of protective effect of *E. littorale* methanolic extract against H_2O_2 induced apoptosis of islets of langerhans.

4.1. Review of literature

Reactive oxygen species (ROS) are constantly generated under normal conditions as a consequence of aerobic metabolism. ROS include free radicals such as the superoxide anion ($O_2^{\cdot-}$), hydroxyl radicals ($OH^{\cdot}$) and the non radical hydrogen peroxide (H_2O_2). They are particularly transient species due to their high chemical reactivity and can react with DNA, proteins, carbohydrates and lipids in a destructive manner which leads to various pathophysiological diseases like Parkinson's, Alzheimer, diabetes, retinal degeneration etc.

Glucotoxicity, leading to chronic hyperglycemia in diabetics involves oxidative stress as an important mechanism for inducing various metabolic complications including insulin resistance. Pancreatic islets seemed to be very sensible to oxidative stress since it expresses a very low amount of anti-oxidant enzymes (Grankvist et al., 1981; Tiedge et al., 1997), like superoxide dismutases (SOD-1, SOD-2), catalase, and glutathione peroxidise (GPx). In contrast, gene expression of the catalytic subunit of γ-glutamylcysteine ligase (GCLC), the rate-limiting enzyme for glutathione (GSH) synthesis, is well expressed in islets (Tran et al., 2004). GSH is the major intrinsic antioxidant in cells. Levels of GCLC mRNA are comparable to those found in liver and greater than those found in muscle, lung and fat (Tran et al., 2004). It has been reported, however, that long-term exposure to high glucose concentrations decreases GCLC expression in mesangial as well as retinal cells, and that this is associated with a decrease in GSH levels (Catherwood et al., 2002; Lu et al., 1999).

Many clinical studies documented chronic oxidative stress in type 2 diabetes are subjected to chronic oxidative stress (Ghiselli et al., 1992; Gopaul et al., 1995; Nourooz et al., 1995; Rehman et al., 1999; Shin et al., 2001; Sakuaba et al., 2002). Murakami et al. (1989) reported that red blood cells from diabetic patients contain low levels of the reduced form of glutathione (GSH), high levels of the oxidized form (GSSG), and a 51% reduction in the GSH/GSSG ratio (Murakami

et al., 1989). Sharma et al. (2000) also reported that type 2 diabetic patients with poor glycemic control had depressed red blood cell GSH levels. After decreasing red blood cell HbA1c levels by sulfonylurea treatment, the red blood cell GSH levels increased twofold, almost reaching the values found in a non-diabetic control group. Similar data were reported by Yoshida et al. (1995) who, in addition, reported that activity of the rate-limiting enzyme of GSH synthesis, c-glutamylcysteine synthase, increased with improved glycemic control. This group also reported that red blood cell thiol transport was significantly and inversely correlated with levels of HbA1c.

Much of the early work addressing molecular mechanisms of glucose toxicity in β cells was performed with the HIT-T15 cell line. Some authors reported that HIT-T15 cells chronically cultured for 6 months in media containing 11.1 mM glucose, a concentration exceeding that necessary to elicit maximal insulin responses, caused loss of insulin mRNA, greatly diminished levels of insulin content, and almost complete disappearance of insulin secretion (Robertson et al., 1992).

It is now well established that the generation or addition of ROS or RNI (reactive nitrogen species) can cause cell death either by apoptosis or necrosis. Apoptosis is a tightly regulated form of cell death characterized by morphological and biochemical changes. These include mitochondrial depolarisation and alterations in phospholipid asymmetry, chromatin condensation, nuclear fragmentation, membrane blebbing, cell shrinkage and the formation of membrane bound vesicles termed apoptotic bodies (Kerr et al., 1972). Many of the morphological changes associated with apoptosis are orchestrated by activation of a cascade of proteases termed caspases (Cohen, 1997; Porter and Janicke, 1999). The caspases are a family of cysteine proteases comprising at least 14 members, all of which contain an active site thiol group necessary for activity (Thornberry and Lazebnik, 1998). This thiol group renders

them particularly susceptible to redox modification by S-nitrosylation or oxidation. Such modifications result in the inhibition of their catalytic activities.

Caspases are also susceptible to oxidative modification by reactive oxygen species such as H_2O_2 and can be readily converted to disulfides and mixed disulfides under conditions of oxidative stress (Fadeel et al., 1998; Shacter, 2000). Caspase-3 activation is often considered as the point of no return in apoptosis and, thus, inhibition of caspases might provide a mechanism to abort the apoptotic cascade. A role for oxidative stress in the induction of apoptosis is provided by studies where the addition of low levels of ROS induces apoptosis and the observation that various antioxidants such as N-acetylcysteine (NAC) can inhibit cell death (Lennon et al., 1991).

In type 1 diabetes, β-cell mass is reduced by 70–80% at the time of diagnosis. Because of the variable degrees of insulitis and absence of detectable β-cell necrosis, it was suggested that β-cell loss occurs slowly over years (Klo¨ppel et al., 1985). These pathology findings are in line with the progressive decline in first-phase insulin secretion in antibody-positive individuals, long before the development of overt diabetes (Srikanta et al., 1983). It was later shown that β-cell apoptosis causes a gradual β-cell depletion in rodent models of type 1 diabetes. Activated macrophages secrete cytokines which are responsible for β-cell apoptosis; probably mediated by three main pathways—namely JNK, ER stress, and liberation of pro-apoptotic proteins from the mitochondria (Kutlu et al., 2003) (Fig. 4.2).

In type 2 diabetic subjects, initial pathological studies suggested a β-cell loss of 25–50% (Kloppel et al., 1985; Clark et al., 1988), but this was debated by others (Guiot et al., 2001). Earlier studies, which matched diabetic patients and control subjects for BMI, showed a significant reduction in β-cell mass (Sakuraba et al., 2002; Butler et al., 2003) and a threefold increase in β -cell apoptosis (Butler et al., 2003). These observations suggest that β-cell mass is decreased in type 2 diabetes,

secondary to increased rates of β-cell apoptosis, but it remains unclear whether this explains the observed functional loss (Kahn, 2003). β -Cell apoptosis may thus be a common feature of type 1 and type 2 diabetes (Fig. 4.1).

Natural flavonoids are emerging as potent therapeutic drugs for free radical-mediated diseases. For example, isoliquiritigenin, baicalein, naringin, and catechin showed neuroprotection through inhibition of ROS, suggesting pharmacologic interest agent for the treatment of Parkinson's disease (Guo et al., 2007; Lee et al., 2005; Singh and Chopra, 2004; Zhan and Yang, 2006). It has been demonstrate that the citrus flavanones hesperidin, hesperetin, and neohesperidin, even at physiological concentrations, have neuroprotective effects against H_2O_2-induced cytotoxicity in PC12 cells. Flavonoids such as butin were investigated for cytoprotective effect against H_2O_2-induced cell damage. Isoflavone puerarin was evaluated for its cytoprotective effect against H_2O_2-induced rat pancreatic islets damage (Fu-Liang et al., 2006). Similarly antioxidant properties and protective effects of a extract of *Hypericum perforatum* on H_2O_2-induced oxidative damage in PC12 cells has been evaluated (Benedí et al., 2004). Some of the medicinal plant extracts viz. *Hypericum perforatum* and isolated compounds like butin and salvinoic acid have been studied to see their protective role on various cells including islets challenged with hydrogen peroxide induced damage.

Enicostemma littorale contains diversified phytochemicals, some of the important constituents of the plant include betulin, a triterpenoid sapogenin, swertiamarin, a secoirridoid glycoside, monoterpene alkaloids like enicoflavine and gentiocrucine. Extract of the plant and its major phytochemical swertiamrin have been reported as antioxidant candidate (Jaishree et al., 2008). EL is known to have good antioxidant activity in diabetic rats (Maroo et al., 2003) as well as in newly diagnosed NIDDM patients (Vasu et al., 2003). In this context, the herb *Enicostemma littorale* was screened for checking its protection against H_2O_2-induced oxidative damage leading to apoptosis on cultured rat pancreatic islets.

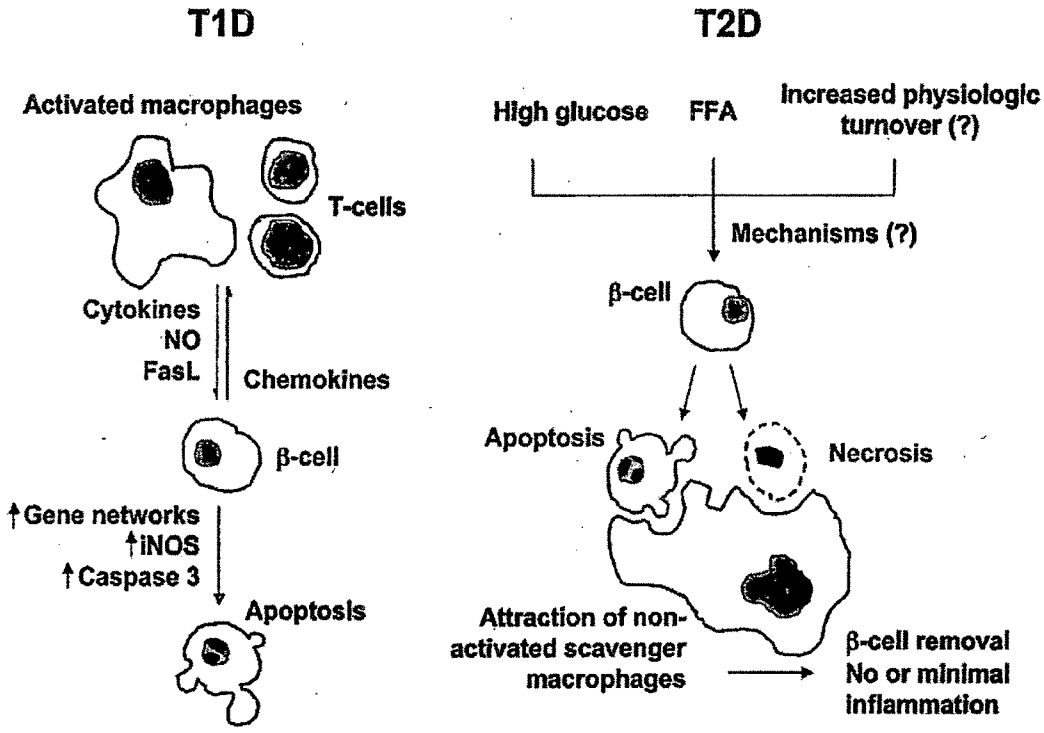


Figure 4.1: Overview of the putative sequence of events leading to β-cell death in animal models of type 1 and type 2 diabetes. T1D, type 1 diabetes; T2D, type 2 diabetes.

4.2. Experimental Design

Islets were isolated from normal rats by the method of Xia et al. (1993), and then subjected to purification. Islets were handpicked under microscope (approx 1000 islets/group). Purified islets were incubated with cumin H_2O_2 at a dose of 50μM for 30 minutes then centrifuzed at 1000 rpm for 10 mins and washed thrice with PBS and used for measurement of different parameters. Another group of islets were pre-incubated with methanolic extract of EL then subjected to H_2O_2 exposure. Dose and time dependent study was carried out with extract. The viability of the islets was checked by trypan blue dye exclusion test (Warburton and James 1995) using 0×4% (W/V) Trypan blue. Blue stained islets were scored as non-viable and the unstained were scored as viable islets. Protection of islets

against oxidative damage which was measured by parameters like fluorescent stains *viz.*, DCF and DAPI, estimation of caspase 3 as an important apoptotic marker. Important antioxidant enzymes *viz.*, SOD, catalase, GSH and GPx were also evaluated. Effect of herbal extract (EL extract) against H_2O_2-induced DNA fragmentation was observed by performing Comet assay. For comet assay islets of langerhans were subjected mild trypsin digesion to obtained single cell suspension. Single cells suspension then exposed to H_2O_2 treatment for 30 mins with and without EL preincubation.

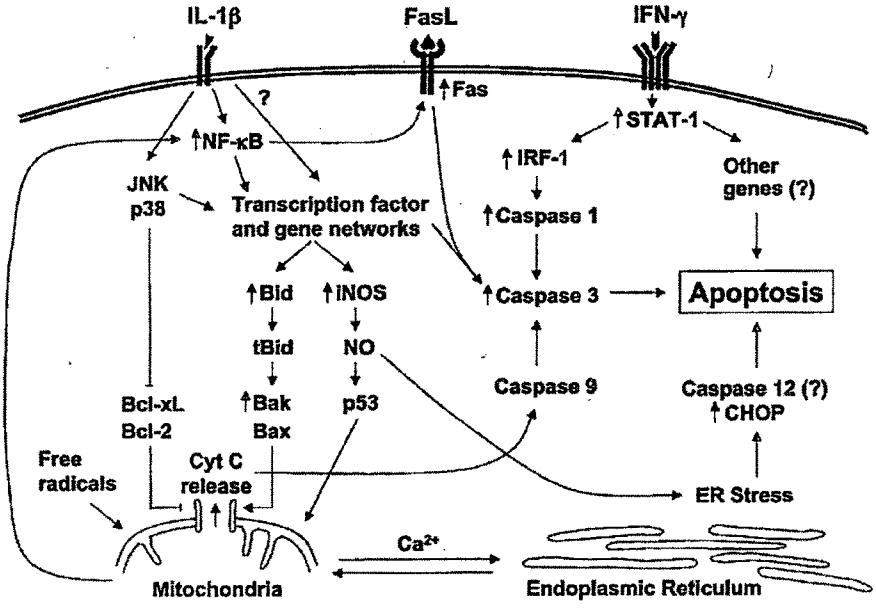


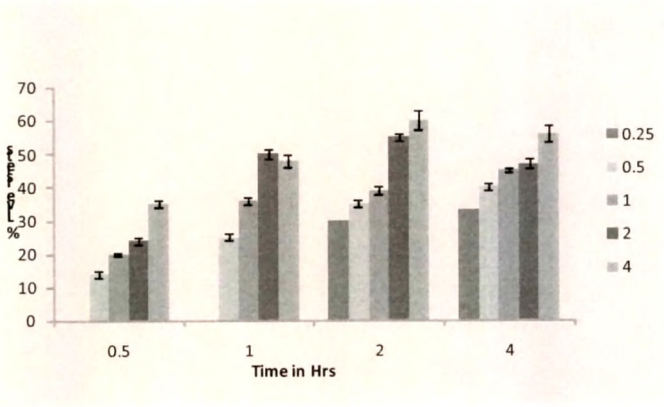
Figure 4.2: Proposed model for the different pathways contributing to the execution of cytokine-induced β-cell apoptosis.

Arrows indicate genes for which expression was modified by cytokines in a time course microarray analysis (Kutlu et al., 2003). β-Cell apoptosis is probably mediated by three main pathways—namely JNK, ER stress, and liberation of pro-apoptotic proteins from the mitochondria.

4.3. Results

4.3.1. Effect of EL on H_2O_2-induced loss of islets viability

In order to assess the cytoprotective effect of EL on islet of langerhans against the oxidative stress, trypan blue viability test was conducted. As seen in Fig. 4.3, treatment with 50 µM H_2O_2 for 0.5 h (30 mins) resulted in viability loss. Preincubation with EL (0.25, 0.5, 1, 2 and 4 mg/ml of assay volume) for above time points; partially protected the islets from the toxicity of H_2O_2 in a dose dependent & time dependent manner.



n = 3

Figure 4.3: Dose dependent and time dependent study of EL on islets of langerhans exposed to 50 µM H_2O_2.

4.3.2. Effect of EL on H_2O_2-induced apoptosis in isolated islets

The percentage of apoptosis was analyzed quantitatively by PS/PI dual staining (Fig. 4.4A). A significant increase in the apoptotic rate was observed after the islets were treated with 50 µM H_2O_2 for 0.5 h. Pretreatment with 2 mg/ml of EL significantly inhibited H_2O_2-induced apoptosis.

4.3.3. Comet Assay

Damage to cellular DNA induced by H_2O_2 exposure was detected by using an alkaline comet assay. The exposure of cells to H_2O_2 increased the comet

parameters of tail length as shown in Fig. 4.4. Treatment with EL decreased the comet tail length, indicating a protective effect of EL on H_2O_2-induced DNA damage (Fig 4.4B).

4.3.4. Caspase-3 Activity

Elevation of caspase 3 enzyme activity was observed in H_2O_2 treated islets of langerhans and was reduced by EL treatment by 42%. (Fig: 4.5)

4.3.5. Effect of EL on H_2O_2-induced reactive oxygen species in isolated islets

Intracellular reactive oxygen species were detected by fluorescein-labeled dye, DCF-DA. As seen in Fig. 4.4.C, 50 µM H_2O_2 significantly increased the fluorescent intensity of reactive oxygen species in islet cells. Pre-incubation with EL (2mg/ml) for 2 h significantly decreased the fluorescent intensity in a dose-dependent manner.

4.3.6. Effect of EL on the antioxidant enzyme activities in isolated islets

As seen in Fig. 4.6, 4.7, 50 µM H_2O_2 significantly (101%) increases lipid peroxidation levels and significantly decreases GSH level (75%). These islets also showed reduced (43%) SOD, catalase (53%) and GPx (44%) activities (Fig 4.8, 4.9 & 4.10). Pre-incubation with EL for 2 h decreases lipid peroxidation by 58% and increases GSH content by 68%. Pre-incubation with EL for 2 h also increased SOD, catalase and GPx activities by 55%, 51% and 44% respectively (Fig 4.8, 4.9 & 4.10).

4.4. Discussion

Diabetes mellitus (DM) is a group of metabolic disorder with different underlying etiologies, characterized by hyperglycemia due to underutilization of

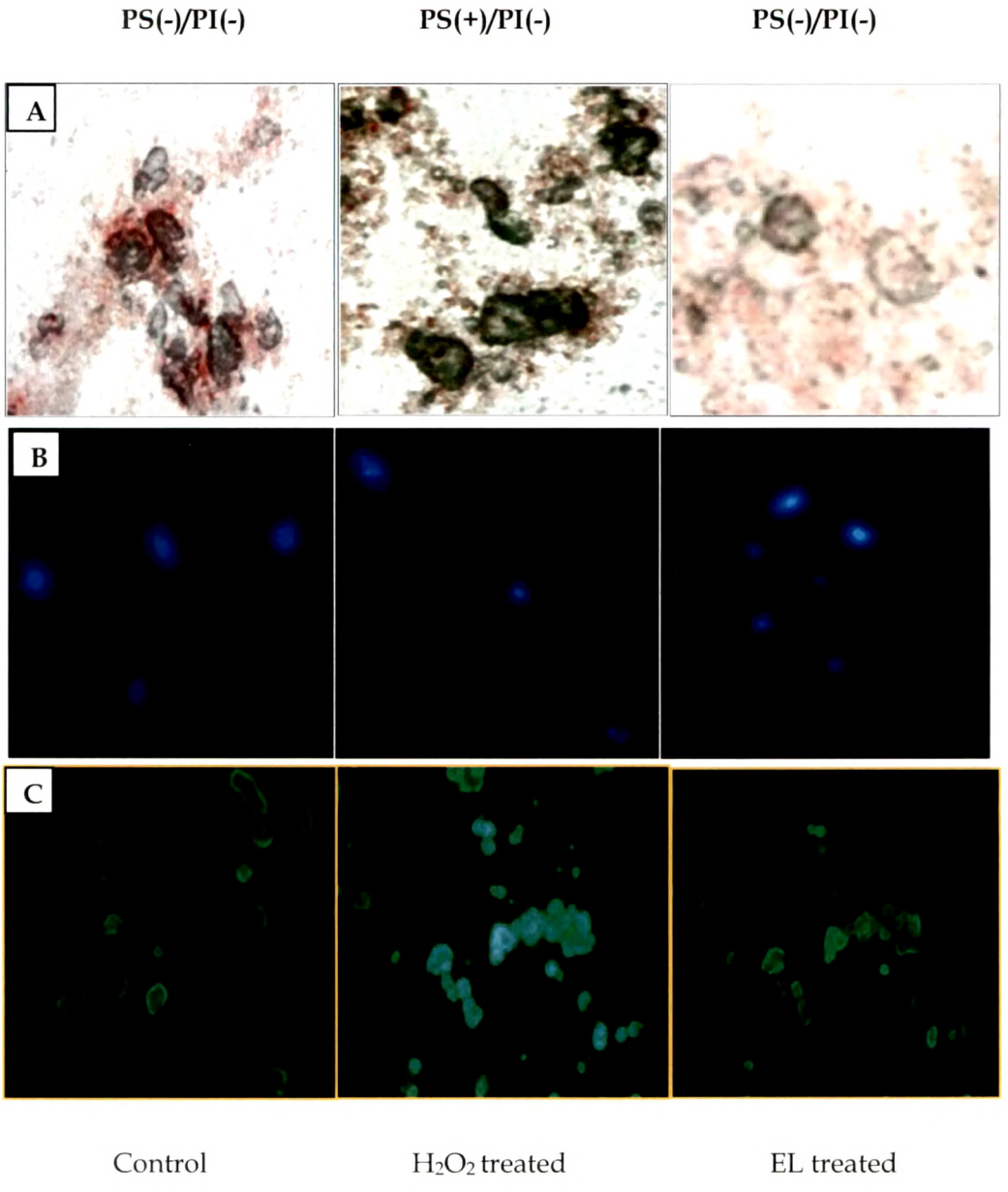


Figure 4.4: Effect of EL treatment on PS/PI staining (A), DNA tailing in comet assay (B), DCF-DA fluorescence staining (C) of islets of Langerhans exposed to 50 μM H_2O_2.

Figure 4.5: Effect of EL on **Caspase-3** activity in H_2O_2 exposed islets

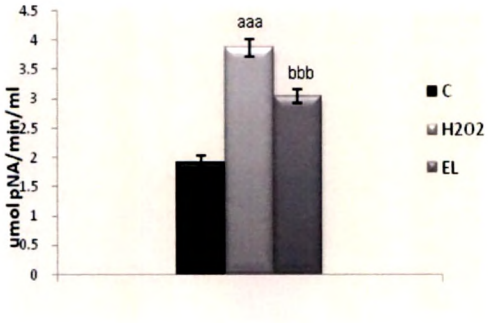


Figure 4.6: Effect of EL on **lipid peroxidation** of islets of langerhans exposed to H_2O_2.

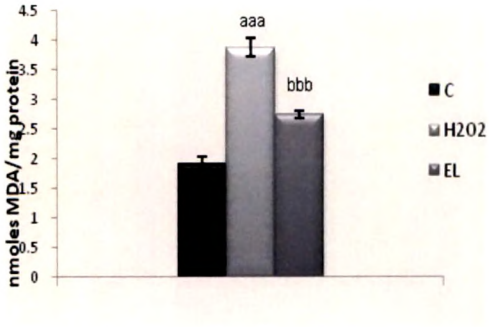


Figure 4.7: Effect of EL on **reduced glutathione** of islets of langerhans exposed to H_2O_2.

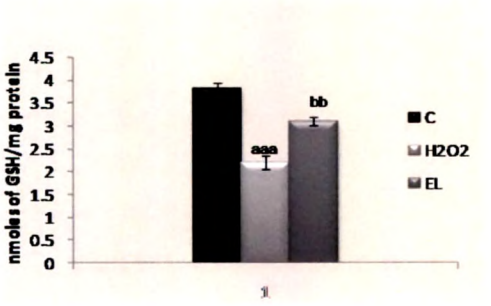


Figure 4.8: Effect of EL on **superoxide dismutase** activity of islets of langerhans exposed to H_2O_2.

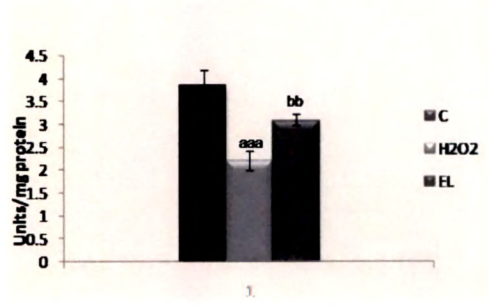


Figure 4.9: Effect of EL on **catalase** activity of islets of langerhans exposed to H_2O_2.

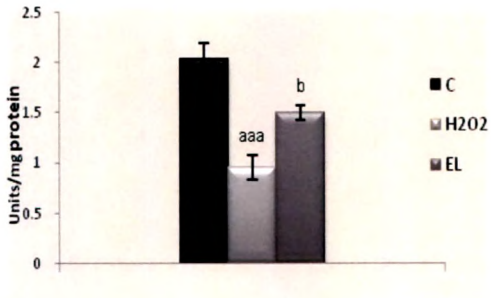
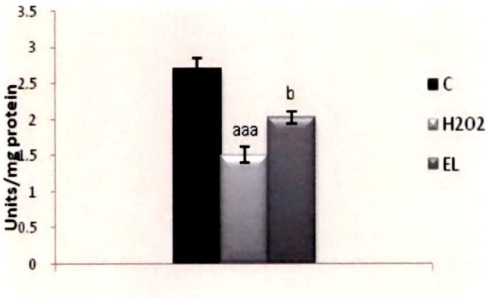


Figure 4.10: Effect of EL on **glutathione peroxidase** activity of islets of langerhans exposed to H_2O_2.



Values are expressed as mean ± SEM (n=6 in each group). a, P<0.05, aa, P<0.01, aaa,P<0.001 vs. C, b,P<0.05, bb P<0.01, bbb, P<0.001 vs. F.

glucose. Under hyperglycemia, the increased blood level of various reducing sugars promotes protein glycation through the Maillard reaction, which consecutively produces Schiff bases, Amadori products and advanced glycation end products (Kiyoko et al., 2003). Reactive oxygen species are formed in this process and trigger tissue damage. Due to intrinsically very low levels of antioxidant enzyme expression and activity (Tiedge et al., 1997; Sigurd et al., 1996), islets are particularly at risk for reactive oxygen species-induced damage as compared with other tissues. Excessive reactive oxygen species may lead to necrosis or apoptosis of islets (Tanaka et al., 2002; Robertson et al., 2003). Treatment of antioxidant chemicals, such as cysteines or N-acetylcysteine, provides some protection to β-cells both in vitro and in vivo (Tanaka et al., 1999; Rasilainen et al., 2002).

EL has been used for the therapy of diabetes mellitus in India as a folk medicine. EL can improve the insulin secretion, decreases insulin resistance (Maroo et al., 2002; Vasu et al., 2003) and also decreases oxidative stress by improving antioxidant status in diabetic animals (Maroo et al., 2003). It is also having hypolipidemic effect (Vasu et al., 2005). These beneficial effects may contribute to its antidiabetic activity. Swertiamarin is a secoiridoid glycoside present in EL, has been reported for its number of activity namely, hepatoprotective, antiedematogenic, free radical scavenging activity, antispastic activity (Vaijanathappa and Badami, 2009). However, little is known about the direct effect of EL on β-cells against oxidative stress induced apoptosis.

In the present study, islets exposed to 50 μM H_2O_2 showed apoptosis. Preincubation with EL (2mg/ml of assay) for 2 h caused suppression of the β-cell death induced by H_2O_2. H_2O_2 was used instead of known β-cell-specific toxins such as alloxan or streptozotocin because H_2O_2-induced apoptosis is a well-established model of a biologically active oxygen-derived intermediate and has been used extensively in the study of reactive oxygen species. Many researches (Krippeit et al., 1994; Maechler et al., 1999; Peter et al., 1999; Tang and Zhang,

2000) showed that transient exposure to H_2O_2 caused a significant damage in islet cells. Our study also showed that 50 μM H_2O_2 increased the reactive oxygen species, which resulted in the damage of islet cells.

Caspase-3 acts as an apoptotic executor and can be activated by H_2O_2. In this study, activities of caspase-3 with EL treated cells were clearly lower than those of untreated cells. Hydrogen-peroxide-associated ROS-mediated DNA damage takes place via direct attack on chromosome or mitochondria pathways, leading to necrosis or apoptosis. Oxidative stress-induced cellular death can be prevented by blocking DNA damage. Our present results suggest that the EL protected H_2O_2-induced cells from apoptosis by blocking DNA damage.

In pancreatic islets, antioxidant enzymes CAT, SOD and GPx genes are expressed at low levels. The high expression levels of catalase and SOD in insulin secretion cells through genetic engineering provide protection against the toxicity of reactive oxygen species (Rita et al., 2002; Markus et al., 1998). In the present study, significant changes of GSH content, SOD, CAT and GPx activities were found at 0.5 h after H_2O_2 addition compared with normal control. Therefore, elimination of H_2O_2 is critical for reducing oxidative stress. The pivotal cellular enzymes for eliminating H_2O_2 are catalase and GPx. In the present study, CAT, SOD and GPx activity were improved in cells along with increased GSH content with EL pre-incubation to islets exposed to H_2O_2. Improvement in antioxidant enzyme activities suggests that the EL strengthened the ability to combat oxidative stress of islet cells.

In summary, the present study provides the evidence that EL can protect pancreatic islets from H_2O_2-induced damage by stimulating the activities of antioxidant enzymes and therefore decreasing DNA damage, but the underlying molecular mechanism remains to be clarified. Our data favour the view that the protective effect of EL may be due to its antioxidant activity.

4.5. Summary

Progressive loss of β-cells is major area of concern in type 1 and type 2 diabetes. Oxidative stress is one of the causal factors for induction of apoptosis of islets of langerhans, responsible for reduction of islet mass in diabetic condition. H_2O_2-induced apoptosis is a well establish method to study the cytoprotective effect of any antioxidant compound. In our study we confirmed the apoptosis event in islets of langerhans upon exposure to H_2O_2 by PS/PI staining, Caspase-3 activity, DNA tailing in comet assay. Cells exposed to H_2O_2 also showed high fluorescence with DCF-DA fluorescence dye; indicator of cell's total oxidative stress. Upon H_2O_2 treatment cell's antioxidant defense system goes down and unable to reverted back apoptotic event as indicated by increase in Caspase-3 activity. Pre-treatment with EL extract decreases fluorescence in the cells, indicating reduced oxidative stress in these cells and also showed improvement in the antioxidant defense system. This improvement in the antioxidant defense system revert back the apoptotic events by decrease in DNA damage as well as decrease in Caspase-3 activity. Thus, our results clearly demonstrated that EL extract is able to protect islets of langerhans from oxidative stress induced apoptosis.

4.6. References

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