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POTENTIAL ROLE OF ROSMARINIC ACID ON BIOMARKERS OF OXIDATIVE STRESS AND ACETYLCHOLINESTERASE IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

Doctoral Thesis

Nadia Mushtaq

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"Dedicated to my dear spouse and family

who always there to back me up; for better or for worse.

Thank you so much for believing in me"

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ABSTRACT

Thesis of Doctor's Degree
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Biochemistry & Toxicology, Federal University, Santa Maria, RS, Brazil.

Potential role of rosmarinic acid on biomarkers of oxidative stress and acetylcholinesterase in streptozotocin-induced diabetic rats

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Place of the defense: Santa Maria, April, 22nd, 2013.

Oxidative stress plays an important role in diabetic pathogenesis. Rosmarinic acid (RA) was used for the first time as an antioxidant agent for inhibition of diabetic nephropathy. Oxidative stress induced by Streptozotocin (STZ) has been shown to damage pancreatic beta cell and produce hyperglycemia in rats, inducing diabetes. In the present study, an attempt was made in investigation, the efficiency of rosmarinic acid in preventing alteration of oxidative parameters in liver, kidney and acetylcholinesterase (AChE) in brain of diabetic rat induced by STZ. The animals were divided into six groups (n=8): control; ethanol; RA 10 mg/kg; diabetic; diabetic/ethanol; diabetic/RA 10 mg/kg.In diabetes, the brain region become affected and showed increased level of lipid peroxidation in hippocampus, cortex and striatum, compared with control. The increased in lipid peroxidation was decreased or maintained to the level of control by RA in hippocampus (28%), cortex (38%) and striatum (47%) of diabetic rats after 21 days treatment at the dose of 10 mg/kg body weight. Furthermore, we found that diabetes caused significant decreased in the activity of antioxidant enzymes i.e. superoxide dismutase (SOD), catalase (CAT), Delta-aminolevulinic acid dehydratase (ALA-D) and non- enzymatic parameter like ascorbic acid, non protein-thiol (NPSH) in liver and kidney. The diabetic group treated with RA (10 mg/kg body weight for 21 days) significantly increased the activity of enzymes SOD, CAT, ALA-D and non-enzymatic ascorbic acid, NPSH in liver and kidney. Furthermore, these results indicate that rosmarinic acid sigificantly mimic the oxidative stress produced during hyperglycemia in STZ-induced diabetic rats. In addition, rosmarinic acid is potential candidate in the prevention of any alteration of pathological condition in diabetes. We suggest that rosmarinic acid could be a suitable candidate for the treatment of diabetes.

Keywords: Streptozotocin;Diabetes; Lipid peroxidation; Acetylcholinesterase; Rosmarinic acid; Liver; Kidney; Rats.

Resumo

Papel potencial do ácido rosmarínico sobre biomarcadores de estresse oxidativo e acetilcolinesterase de ratos diabéticos induzidos por estreptozotocina

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O estresse oxidativo desempenha um papel significativo na patogênese do diabetes. O ácido rosmarínico (RA) foi utilizado pela primeira vez como agente antioxidante para a inibição da nefropatia diabética. O diabetes induzido por estreptozotocina (STZ) é capaz de destruir as células beta pancreáticas e produzir hiperglicemia causando estresse oxidativo. No presente estudo, investigou, a eficiência do ácido rosmarínico na prevenção de alteração de parâmetros oxidativos no fígado, rim e acetilcolinesterase (AChE) no cérebro de ratos diabéticos induzidos por STZ. Os animais foram divididos em seis grupos (n = 8): controle; etanol; RA 10 mg / kg; diabéticos; diabéticos /etanol; diabético / RA 10 mg / kg. Ratos diabéticos apresentaram um aumento do nível de peroxidação lipídica no hipocampo, córtex e estriado, em comparação com o controle. O tratamento com ácido rosmarínico (10 mg/kg) durante 21 dias preveniu o aumento da peroxidação lipídica no hipocampo (28%), no córtex (38%) e no estriado (47%) de ratos diabéticos. Além disso, o diabetes causou uma diminuição significativa na atividade das enzimas superóxido dismutase (SOD), catalase (CAT) e delta aminolevulínico-desidratase (ALA-D) e nos níveis dos antioxidantes não-enzimáticos ácido ascórbico e tióis não-proteicos (NPSH) no fígado e no rim. O tratamento com ácido rosmarínico preveniu o decréscimo na atividade da SOD, CAT e ALA-D e o decréscimo nos níveis de ácido ascórbico e NPSH no fígado e no rim. Assim, os resultados encontrados neste estudo indicam que o ácido rosmarínico diminuiu o estresse oxidativo produzido pela hiperglicemia em ratos diabéticos induzidos por STZ. Dessa forma, é plausível sugerir que o ácido rosmarínico é um potencial candidato na prevenção de alterações no sistema colinérgico bem como de danos oxidativos observados no diabetes.

Palavras chaves:Estreptozotocina; Diabetes; Peroxidação lipidica; Acetilcolinesterase; Ácido rosmárinico; Fígado; Rim; Ratos;

LIST OF ABBREVIATIONS

AChE- **Acetylcholinesterase**

ALA-D- **Delta-aminolevulinic acid dehydratase**

CAT – **Catalase**

DM – **Diabetes mellitus**

DNPH- **Dinitrophenyl hydrazine**

GSH- **Glutathione peroxidase**

H_2O_2-**Hydrogen peroxide**

MDA- **Malondialdehyde**

NPSH- **Non protein-thiol**

PBG- **Porphobilinogen**

RA- **Rosmarinic acid**

ROS- **Reactive oxygen species**

SDS- **Sodium dodecylsulfate**

SOD- **Superoxide dismutase**

STZ – **Streptozotocin**

TBA- **Thiobarbituric acid**

TBARS- **Thiobarbituric acid reactive substances**

TCA- **Trichloroacetic acid**

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1. Introduction

Third world countries including Brazil and Pakistan are now facing persisting emerging epidemics of chronic diseases including diabetes mostly due to increased urbanization, westernization, high consumption of industrialized foods and physical inactivity (WILD et al., 2004). Diabetes is the third leading fatal disorder after cancer and heart disease (ANDALLU et al., 2002). It is a group of metabolic disorders characterized by high blood glucose levels (hyperglycemia) resulting from defects in insulin secretion, insulin action or both (AMERICAN DIABETES ASSOCIATION, 2009). Insulin is a hormone produced by specialized beta cells of the pancreas, its function is to monitor the glucose level in blood as both extremes are dangerous and can disturb the body's chemical processes. Glucose provides energy to cells for normal functions.

Hyperglycemia a hallmark of diabetes, contributes to the development and progression of diabetes often accompanied by glycosuria, polydipsia, polyuria, weight loss, sometimes with polyphagia, and blurred vision (SAILAJA et al., 1993; CELIK et al., 2002). Impairment of growth and susceptibility to certain infections may also accompany chronic hyperglycemia.

Diabetes is a multifactorial disease, associated with both microvascular (retinopathy, nephropathy and neuropathy) and macrovascular (ischemic heart disease, peripheral vascular disease, and cerebrovascular) complications (SAYDAH et al., 2004). These complications can result in significant morbidity and mortality in people with diabetes (LOCKMAN et al., 2011).

According to WHO (2003) the classification of diabetes includes four clinical classes.

Type 1 Diabetes: Type 1 diabetes is a lifelong condition in which the body can't control the amount of glucose in the blood. According to the American Diabetes Association

(2010) almost 10% of diagnosed cases are type 1. Diabetes Type 1 is also known as an autoimmune disease (LERNMARK et al., 2000). In this case body does not produce insulin. Insulin medication (usually by injection) is necessary to provide the body with insulin, and thus type 1 diabetes is described as insulin-dependent diabetes. The condition is usually first seen in childhood or adolescence and so is often called juvenile diabetes (COOK et al., 2008).

Type 2 Diabetes: affects approximately 90% of the diabetic population (AMERICAN DIABETES ASSOCIATION, 2010). In type 2 diabetes either the body does not produce enough insulin or the cells ignore the insulin to maintain a normal glucose level (LEBOWITZ et al., 1999; VOTEY, 2007). This is known as insulin resistance (TAYLOR, 2012). Unlike type 1 diabetes, patients with type 2 diabetes do not usually require insulin but most patients require oral medication to lower blood glucose levels. Although type 2 diabetes typically affects individuals older than 40 years, but it has also been diagnosed in children as young as 2 years of age who have a family history of diabetes (VOTEY, 2007). Most patients of diabetes type 2 are obese, and obesity itself causes some degree of insulin resistance (ADA, 2008). Type 2 diabetes can be managed by diet, exercise and healthy life (AMERICAN DIABETES ASSOCIATION, 2005).

Other specific types of diabetes: It is due to some other causes, e.g., genetic defects in β-cell function or in insulin action, diseases of the exocrine pancreas (such as cystic fibrosis), and drug- or chemical-induced (such as in the treatment of HIV/AIDS or after organ transplantation) (CANADIAN DIABETES ASSOCIATION, 2003).

Gestational diabetes mellitus (GDM): Gestational diabetes mellitus (GDM) is defined as any degree of glucose intolerance with onset or first recognition during pregnancy (FRASER & HELLER, 2007). In most cases, gestational diabetes is managed by diet

and exercise and goes away after the baby is born. Very few women with gestational diabetes require insulin to control this type of diabetes.

Worldwide rapid increase in diabetes incidence is one of the bases for the growing interest in the use of experimental diabetic models including streptozotocin (STZ) or alloxan (Figure 1). These experimental models are essential tools for understanding the molecular basis, the pathogenesis of complications and the utility of therapeutic agents in diabetes (CHEN & WANG, 2005). STZ is a compound derived from *Streptomyces achromogenes,* which enters pancreatic β cells through glucose transporter 2 (glut2) channels in the plasma membrane and causes cellular toxicity and local immune responses that lead to hypoinsulinemia and hyperglycemia in animals (SZKUDELSKI, 2001). STZ is known for its specific toxicity associated with pancreatic β-cells (NTP, 2005). Experiments demonstrate that doses of STZ in the range of (40 mg/kg, 60 mg/kg i.p. or i.v.) in rats results in hyperglycemia within 72 hours (CASEY et al., 2004; SHAH et al., 2006; SINGH et al., 2006; GOJO et al., 2007; AL-QATTAN et al., 2008).

It is hypothesized that the diabetogenic action of STZ in animals is mediated through a reduction of nicotinamide adenine dinucleotide (NAD) in pancreatic cells (WEISS, 1982). The DNA damage caused by STZ mediated alkylation is repaired by an excision repair process, which requires the activation of the NAD dependent enzyme poly (ADP-ribose) synthetase (TAKAMA et al., 1995). It is postulated that in the beta cell this enzyme is continuously activated, thus depleting the cellular NAD. The critical loss of NAD leads to a cessation of cellular function and eventually cell death.

STZ administration produces toxic radicals (oxygen free radicals), including hydroxyl and carbonium radicals (SOBREVILLA et al., 2011). Moreover, it is also speculated that intraperitoneal injection of STZ into rats induced a significant decrease

in antioxidant enzymes activities which further results in damage of DNA, proteins and lipids (DUZGUNER & KAYA, 2007; HAMDEN et al., 2008; LEI et al., 2008) and mitochondrial dysfunction (RAZA et al., 2004).

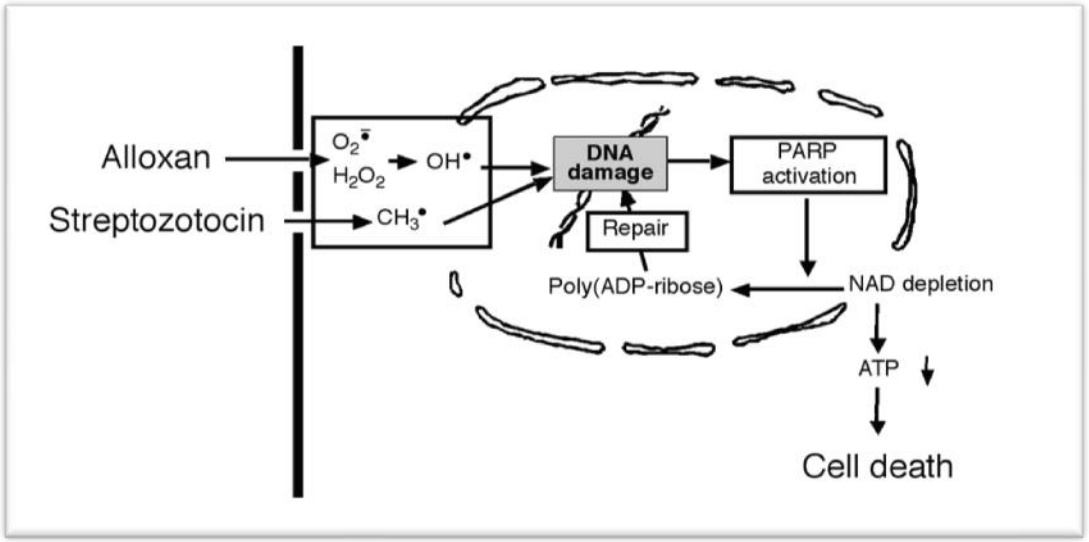

Figure.1 A unifying model for the action of diabetogenic agents, streptozotocin and alloxan (Okamoto & Takasawa, 2003)

Human body produces oxygen free radicals (superoxide and hydroxyl radicals) and other reactive oxygen species (ROS) (hydrogen peroxide, nitric oxide, peroxynitrile and hypochlorous acid) by several different biochemical processes. The oxygen free radical is characterized by having unpaired electron in its molecular structure. They are short lived and highly reactive for example H, O and singlet oxygen (WINTERBOURN, 2008).

Oxidative stress results from an imbalance between radical-generating and radical-scavenging systems i.e. increased free radical production and/ or failure of antioxidant defense (UTTARA et al., 2009). Oxidative stress, is the unifying link between the various molecular disorders in diabetes (EVANS et al., 2002). The presence of oxidative stress may be verified in one of three ways: (1) direct measurement of the ROS (2) measurement of the resulting damage to biomolecules

DNA, proteins, carbonyl etc. and (3) detection of antioxidant levels (HALLIWELL & WHITEMAN, 2004).

Among the biological molecules, lipids are most susceptible to the attack of ROS and nitrogen species (NIKI et al., 2005). Lipid peroxides are the products of the chemical damage done by oxygen free radicals to the lipid components of cell membranes (DIANZANI & BARRERA, 2008). These polyunsaturated fatty acids, containing two or more double bonds, are particularly vulnerable to peroxidation, and once the process is initiated, it proceeds as a free radical–mediated chain reaction involving initiation, propagation, and termination (GAGO-DOMINGUEZ et al., 2005).

Lipids when react with free radicals, they undergo peroxidation to form lipid peroxides, which decompose to form numerous products including malondialdehyde (MDA) (KOSE & DOGAN, 1995; CATALA, 2006). MDA is formed during lipid peroxidation as end product after rupture of the carbon chain of unsaturated fatty acid and reacts with amino groups of enzymes, proteins and DNA. Its assessment is considered as a reliable marker of oxidative damage. The end-product MDA reacts with deoxyadenosine and deoxyguanosine in DNA, forming DNA adducts to them (WANG et al., 2004). Lipid peroxides decrease membrane fluidity and change the activity of membrane-bound enzymes and receptors (HALESTRAP et al., 2002). Studies revealed that increased levels of lipid peroxides have been implicated in the pathogenesis of diabetic complications (MAHBOOB et al., 2005; SINGH et al., 2009; VARASHREE et al., 2011).

Furthermore, it is reported that high level of LPO is responsible for the formation of lipid hydroperoxides in membrane, which lead to alteration of membrane-bound enzymes like acetylcholinesterase (AChE) (MEHTA et al., 2005). It is also postulated that increased lipid peroxidation products, such as 4-HNE contribute to

neuronal loss in conditions associated with oxidative stress (KUTUKA et al., 2004), which causes learning and memory disorders because lipid peroxidation not only alters membrane lipids milieu but also contribute to the development of chronic complications in the central nervous system (YUN-ZHONG et al., 2002).

One of the most important mechanisms is responsible for correct cholinergic function is performed by AchE, an efficient enzyme of nervous system. AchE hydrolyzing predominantly choline esters, and characterized by high concentrations in brain, nerve and red blood cells (RBCs) regulates cholinergic nerve and neuromuscular transmission (ALLAM et al., 2007). Increase in AChE activity has been associated to enhancement in the degradation of acetylcholine and reduces cholinergic transmission in diabetes (XIE & DU, 2005). Diabetes- induced oxidative damage is responsible for dysfunction of neurotransmitters (RORIZ-FILHO et al., 2009), which is secondary to the metabolic disorders such as hyperglycemia and acidosis.

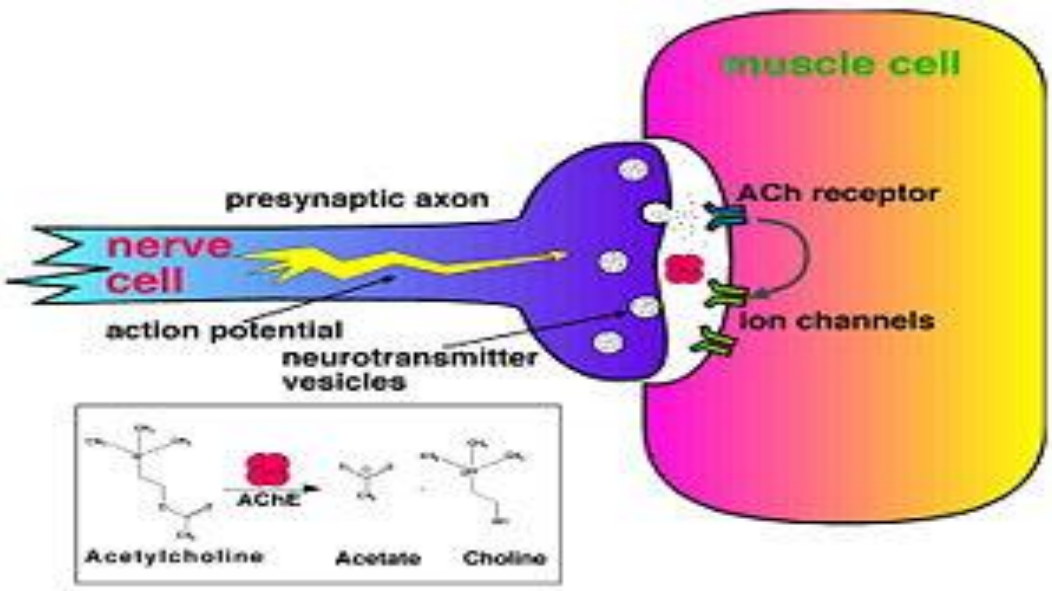

Figure 2.The mechanism of action of acetylcholinesterase.
(http://www.proteopedia.org/wiki/index.php/Acetylcholinesterase)

Several studies suggest that hyperglycemia leads to neurological dysfunction and injury (STRACHAN et al., 2003; BRANDS et al., 2007). Abnormalities affecting AChE activity has been reported in several diseases including diabetes. Increased acetylcholinesterase activity can reduce the quality and span of memory. It has been revealed that inhibition of AChE is effective in the treatment of these diseases to prolong the effect of ACh on the receptor and may attenuate inflammation by increasing the ACh concentration in the extracellular space (NIZRI et al., 2006). These AChE inhibitors reduce lymphocyte proliferation and the secretion of pro-inflammatory cytokines (KAMAL et al., 2009).

There is an association between increased oxidative stress and lower antioxidant defense which plays important role in the pathogenesis of diabetes (LODOVICI et al., 2008; LIKIDLILID et al., 2010). The term antioxidant may be defined as "any substance exogenous or endogenous in nature that delays or inhibits oxidative damage to a target molecule and protects biologically important molecules such as DNA, proteins, and lipids from oxidative damage and consequently reduce the risk of several chronic diseases (HALLIWELL et al., 2006). Hyperglycemia can generate not only more ROS but also weaken antioxidative mechanism through glycation of the scavenging enzyme (KHAN et al., 2004)

Humans have evolved with antioxidant systems to protect against free radicals. These systems include some enzymatic antioxidants produced in the body (endogenous) and others obtained from diet or non-enzymatic (exogenous). Enzymatic antioxidants are comprised of limited number of proteins such as catalase (CAT), glutathione peroxidase (GSH) as well as superoxide dismutase (SOD) along with some supporting enzymes. Non-enzymatic antioxidants include direct acting antioxidants, which are

extremely important in defense against oxidative stress, such as ascorbic and lipoic acid, glutathione, polyphenols and carotenoids (JAKUS et al., 2000).

Consequences of oxidative stress (Figure. 3) in diabetes has been shown, to change the antioxidant enzymes, non-enzymatic protein glycosylation (VLASSARA et al., 2000), auto-oxidation of glucose (WOLFF et al., 1991), impaired glutathione metabolism, lipid peroxides (DAVI et al., 2005) and decreased vitamin C levels (NIRMALA et al., 2011). Also this is particularly dangerous for the beta islets, which are more susceptible to ROS because of weak antioxidative defense mechanisms (LENZEN et al., 2008). The level of antioxidant enzymes critically enhance the vulnerability of various tissues to oxidative stress and are associated with the development of complications in diabetes (LIPINSKI et al., 2001)

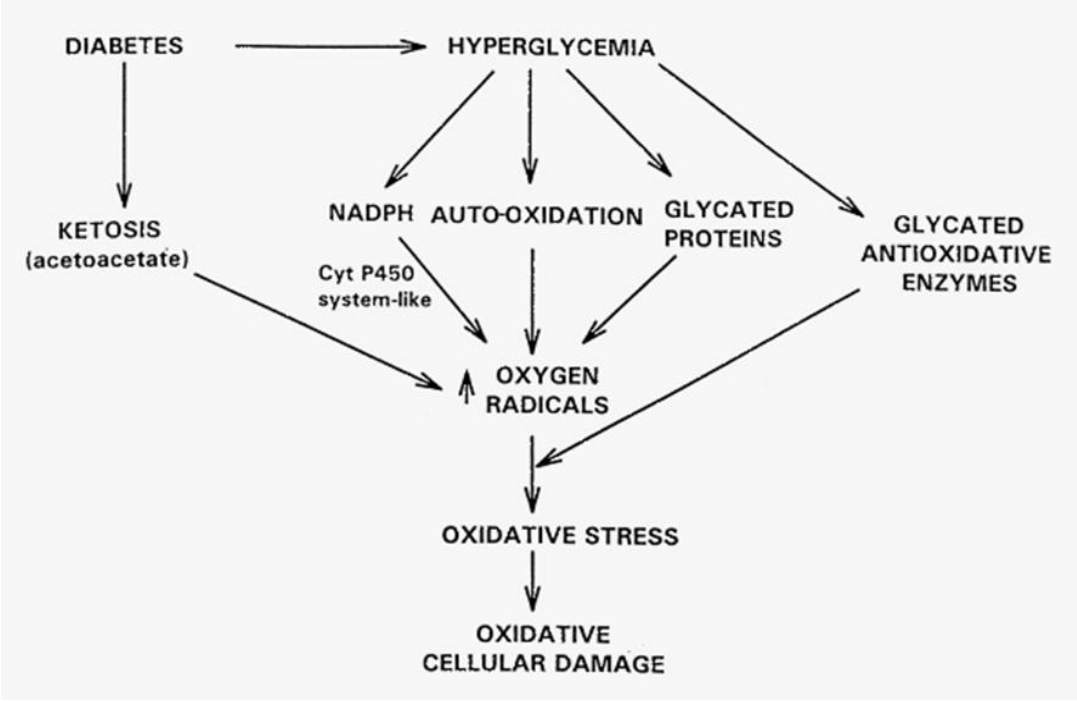

Figure 3. Mechanisms of oxidative cellular damage in diabetes (Jain, 2000).

A family of metalloenzymes known as SOD (EC 1.15.1.1) is the front line of defense against ROS-mediated injury catalyzes the dismutation of superoxide radicals

(NOSRATOLA et al., 2003). SOD discovered by american biochemist Irwin Fridovich and his graduate student Joe McCord in 1969 (MC CORD & FRIDOVICH, 1969). The ubiquitous superoxide is dismutated to a far less reactive product, hydrogen peroxide (H_2O_2) to molecular oxygen and peroxide thus it is critical for protecting the cell against the toxic products of aerobic respiration (PERRY et al., 2010). ‘O2•¯is commonly produced within aerobic biological systems, and SOD provides an important defense against it.

Catalase was first noticed in 1818 when Louis Jacques Thénard, who discovered H_2O_2, suggested that its breakdown is caused by a substance. Later this substance was named as catalase. CAT is a hemeprotein, one of the important antioxidative factors involved in elimination of ROS. It is localized in the peroxisomes or the micro-peroxisomes. One molecule of CAT can catalysis the decomposition of millions of hydrogen peroxide molecules into oxygen and water (KANGRALKAR et al., 2010). It also uses hydrogen peroxide to oxidize potentially harmful toxins in the body including formaldehyde, formic acid, alcohol, and phenol (GARDNER et al., 2003). CAT plays important role in protection of pancreatic β-cells from damage by H_2O_2, which inhibit insulin signaling (GABRIELE et al., 2010). This increased hydrogen peroxide, due to CAT deficiency, plays a role in the complications of DM (GÓTH et al., 2012).

Human body antioxidant system (Figure 4) is incomplete without exogenous reducing compounds such as vitamin C and non protein thiol (NPSH). Vitamin C is hydrophilic antioxidant. Its role is to quench excess oxygen-derived reactive species generated during normal cellular reactions (VALKO et al., 2007; BOUAYED, 2010). Studies have demonstrated that the vitamin C at high doses reduce the accumulation of sorbitol in the erythrocytes of diabetes patients by inhibiting aldose reductase, the enzyme that converts glucose to sorbitol when stored in body, is harmful for nerves

eyes and kidneys (GOODARZI., 2006). Vitamin C may improve glucose tolerance in Type 2 diabetes (RAFIGHIET al., 2013) A decrease in vitamin C is mainly responsible for hyperlipidemia and hypertension in diabetes (WU et al., 2007). Transport of vitamin C into cell is facilitated by insulin. Many diabetics do not have enough intracellular vitamin C due to impaired transport or dietary insufficiency (YAMADA, 2004).

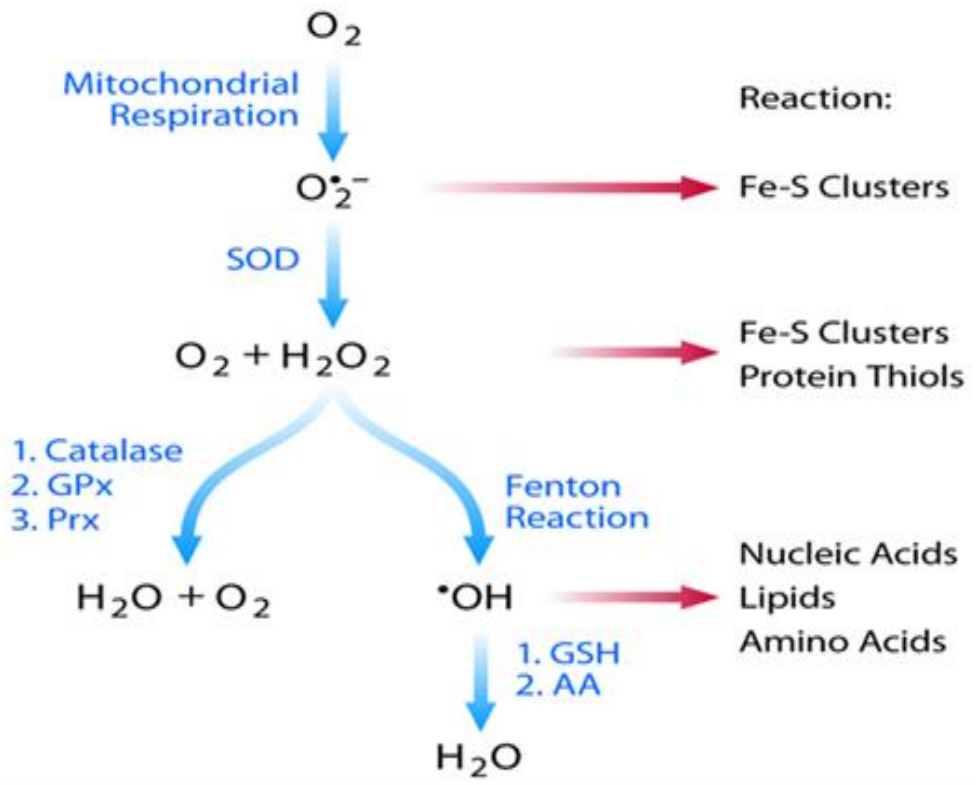

Figure 4.Defense mechanism against damage by ROS (Merksamer et al., 2013).

Glutathione (GSH) (Figure 5) is a major non-protein thiol in living organism, reduced glutathione synthesized mainly in the liver, is an important non-enzymatic antioxidant (CALLUM & JAMES, 2007). Glutathione reductase requires NADPH for its activity, resulting in the reduction of oxidized form of glutathione GSSG to reduced glutathione (GSH) and the corresponding oxidation of NADPH to $NADP^+$.

Deregulation of GSH concentration indicates disease state including diabetes (LIVINGSTONE et al., 2007). Erythrocyte glutathione level become low in diabetes due to impaired activity of the enzyme GCS (γ-glutamylcysteine synthetase) which is involved in the biosynthesis of glutathione (MURAKAMI et al., 1989; LANG et al., 2000). It is an important soluble antioxidant in the brain, detoxifies H_2O_2 and lipid hydroperoxides (CHATTERJEE, 2013). Furthermore, at the same time oxidation of GSH results in DNA fragmentation this ultimately leads to cell death (HIGUCHI, 2004).

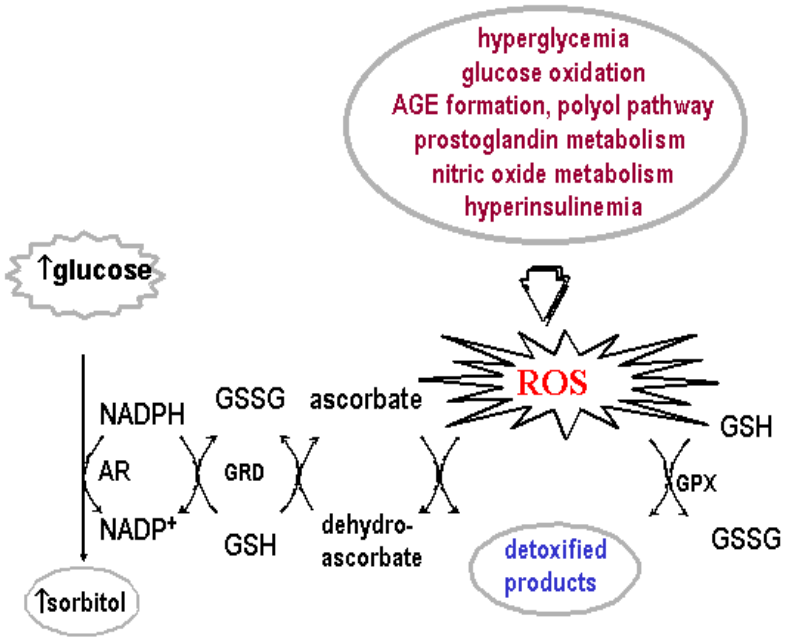

Figure 5. Mechanisms for increased oxidative stress in diabetes mellitus. (Laaksonen & Sen, 2000).

In healthy human body, there should be an approximate balance between production of reactive species and antioxidant defenses. High levels of oxidative stress affect every organ, and have been linked with different diseases including diabetes and

cancer where kidney and liver both are organs highly vulnerable to ROS due to the abundance of long-chain polyunsaturated fatty acids (VIDELA, 2008).

All diabetic patients are considered to be at risk for nephropathy. Diabetes leads to increased glomerular hyperfiltration and glomerular pressure (OZBEK, 2012). This increased glomerular pressure leads to damage to glomerular cells and to development of focal and segmental glomerulosclerosis, which results in the chronic renal failure (QIAN et al., 2008). In this situation kidney antioxidant enzyme activities are found to be reduced in diabetes (SADI et al., 2012).

On the other hand the association between liver disease and diabetes is also well known. Diabetes itself contributes to liver disease, via non-alcoholic fatty liver disease (NAFLD), nonalcoholic steato hepatitis (NASH), cirrhosis, and ultimately hepatocellular carcinoma (MOSCATIELLO et al., 2007). Advanced glycation end products (AGEs) in hyperglycemia damage endothelial cells and lead to capillary wall thickening results in a condition called angiopathy which is another main pathophysiology in liver (HUDACKO et al., 2009).

δ-Aminolevulinic acid dehydratase (ALA-D; EC 4.2.1.24) is a cytosolic sulfhydryl-containing enzyme in the heme biosynthetic pathway that catalyzes the condensation of 2 molecules of 5-aminolevulinic acid to form 1 molecule of the monopyrrole porphobilinogen (PGB) (Figure 6). In the subsequent steps PGB is assembled in to tetrapyrrole molecules which constitute prosthetic groups of physiologically relevant molecules including CAT, hemoglobin and cytochromes. δ-ALA-D is extremely sensitive to oxidizing agents (FARINA et al., 2003). δ-ALA-D inhibition can impair heme biosynthesis and its substrate ALA has been shown to induce pro-oxidant events (TOMÁS-ZAPICO et al., 2002). Number of studies revealed that the activity of δ-ALA-D is inhibited in diabetes (FOLMER et al., 2002; KADE et

al., 2009a) and other diseases related to oxidative stress (SOUZA et al., 2007;BARBOSA et al., 2008; GONCALVES et al., 2009).

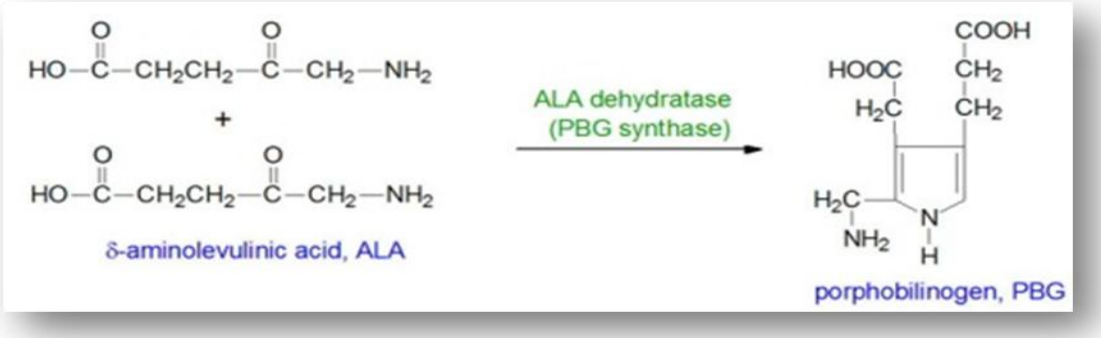

Figure 6. Synthesis of porphobilinogen (PBG) (FLORA et al., 2008).

There are several factors affect the activity of δ-ALA-D. Experiments with diabetic rats demonstrate that δ-ALA-D showed a significant positive correlation with important antioxidants and negative correlation with TBARS, indicating that δ-ALA-D activity is a reliable marker for oxidative stress in diabetes (SCHMATZ et al., 2012).

Compelling evidence has led to the conclusion that the nutrients containing antioxidants are thought to provide protection against different diseases (TENDON et al., 2005; HUY et al., 2008; HAMID et al., 2010). Additionally, there are reports indicating that worldwide, over 1200 species of plants have been recorded as traditional medicine for diabetes and these are the best tool to obtain a variety of newer herbal drugs in the prevention of diabetes (ALARCON-AGUILARA et al 2002; KESARI et al., 2007). This led to sudden increase in the number of herbal drug manufactures (NASREEN & RADHA, 2011). Herbal medicines as the major therapy in traditional system of medicine have been used in medical practices since ancient times. The beneficial medicinal effects of these medicinal plants typically result from the combinations of secondary products present in the plant (BRISKIN, 2000). Polyphenols are the most significant compounds exhibit strong antioxidant activities. The antioxidant

activity of polyphenols is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers and metal chelators (PRIOR et al., 2005; LOPEZ et al., 2007; CIZ et al., 2008; GEBICKA& BANASIAK, 2009).

One of the important polyphenols is the rosmarinic acid attracted much attention since it was identified to be the main compound responsible for the antiviral activity of lemon balm in treating *Herpes simplex* (MAY & WILLUHN, 1978; BORKOWSKI & BIESIADECKA, 1996). Rosmarinic acid is a natural antioxidant found as secondary metabolites. Two Italian chemists, SCARPATI & ORIENTE (1958), isolated it for the first time as a pure compound and named it rosmarinic acid according to the plant *Rosmarinus officinalis*. Rosmarinic acid, together with similar compounds, has been known as "Labiatengerbstoff' even before its chemical structure was elucidated (HERMANN, 1960), as an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid. It is mostly found in Lamiaceae family such as rosemary, sage, lemon balm and thyme, as well as occurs in several taxonomically non-related families of the plant kingdom (PETERSEN & SIMMONDS, 2003). These plants are widely used as culinary herbs, especially in Mediterranean dishes and have long been used in traditional medicine in many countries for the treatment of numerous diseases including diabetes (MAROO et al., 2002). Rosmarinic acid also has a large number of other biological activities such as anti-hyperglycemic (KUMAR et al., 2010), anti-inflammatory, (JIANG et al., 2009), antioxidant (LAMIEN-MEDA et al., 2010) anticancer (SCHECKEL et al., 2008), anti-allergic (LEE et al., 2008) and antiviral (DUBOIS et al., 2008).

The biological effects of rosmarinic acid on health depend on the bioavailability and metabolism (PORRINI & RISO, 2008). Studies on bioavailability of rosmarinic acid in different animal models showed that rosmarinic acid is absorbed, transported,

modified and is well tolerated in skin, blood, bone and muscle while intravenously administered rosmarinic acid was distributed in various tissues such as lung, spleen, heart and liver (RITSCHEL et al., 1989; BABA et al., 2004). Pharmacokinetic studies of rosmarinic acid in rats showed that this polyphenol is well absorbed through the small intestine and reaches full concentration in the blood plasma within 30 minutes. The recovery of intact rosmarinic acid and metabolites in rat urine was 0.077% of the amount ingested (NAKAZAWA & OHSAWA, 1998). Rosmarinic acid is absorbed by both oral and parenteral routes of administration with t-half of about 1.8h; half an hour after i.v. administration (AL-SEREITI et al., 1999). The daily dosage of rosmarinic acid is less clear, since no clinical studies have been done on rosmarinic acid itself. One approach would be to determine the amount of rosmarinic acid that would be present in dried rosemary leaves, the turns out to give a rosmarinic acid dose of about 240 mg/day. Doses higher than this is not unsafe, but requiring caution.

Rosmarinic acid is considered one of the most potent antioxidants among the simple phenolic and hydroxyl cinnamic acids (SOOBRATTEE et al., 2005). Rosmarinic acid displays a strong scavenger activity for $ONOO^-$ and other free radicals (QIAO et al., 2005). The free radical scavenging activity of phenolic compounds is important for their direct antioxidant activity by breaking the free radical chain reactions, inhibiting its initiation and preventing chain propagation (RICE-EVANS et al., 1996; CROFT, 1998).

Structurally rosmarinic acid has two phenolic rings (Figure. 7). The main active groups of rosmarinic acid are the two phenolic hydroxyls in the rings A and B (CAO et al., 2005), in contrast with other flavonoids in which the main active position is in the ring B (SILVA et al., 2002). Like other phenolic compounds rosmarinic acid easily donates a hydrogen atom from an aromatic OH group to a free radical, because it is able

to stabilize an unpaired electron through its delocalization (DUTHIE & CROZIER, 2000). Rosmarinic acid may act as a strong chelating agent. As chelating ability is an important property because it brings about the reduction of the concentration of transition metal that catalyzes lipid peroxidation (PSOTOVÁ et al., 2003).

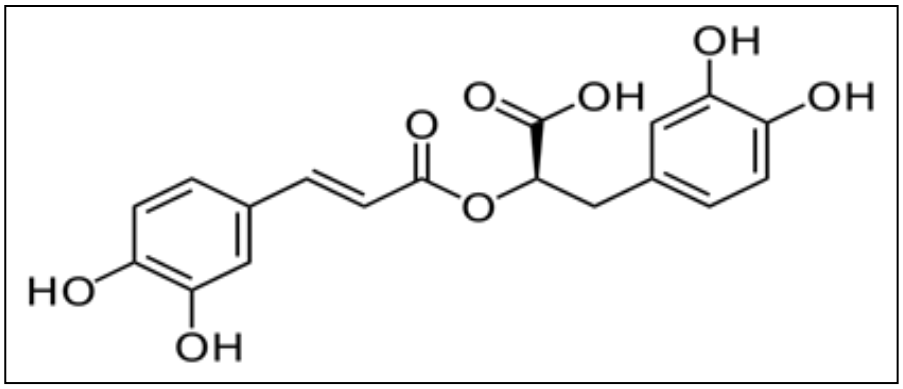

Figure. 7. Chemical structure of rosmarinic acid. 3-(3,4-Dihydroxyphenyl)-1-oxo-2E-propenyl]oxy]-3,4-dihydroxybenzene propanoic acid.

Treatment of diabetes with rosmarinic acid causes a decrease in malondialdehyde (MDA) levels. This decrease in MDA may increase the activity of glutathione peroxidase (GPX) hence cause inactivation of LPO reactions (BAKIREL et al., 2008). Moreover several reports indicate that the compounds responsible for antioxidant activity of *Rosmarinus officinalis* are mainly phenolic acids, such as rosmarinic acid, carnasol, and caffeic acids (KHALIL et al., 2012)

Rosmarinic acid has a therapeutic potential in treatment of many pathological conditions. Rosmarinic acid has been shown to have anti allergic activity by killing allergy-activated T cells and neutrophils during allergic reactions without affecting the T cells or neutrophils in their resting state (SANBONGI et al., 2003). Earlier,

researchers demonstrated that daily treatment with 1.5 mg of rosmarinic acid in perilla leaf extract given orally to mice prevented perennial rhinitis (SANBONGI et al., 2003)

Another way in which rosmarinic acid exhibits positive effect is its neuroprotective role. Studies have demonstrated that rosmarinic acid prevents the aggregation of beta-amyloid plaque in the brain (ALKAM et al., 2007). Rosmarinic acid also shows neuroprotective role to modulate some of the intracellular events (e.g. Ca^{2+} overload, c-fos expression) involved in neuronal death against three different harmful stimuli: oxidative stress, excitotoxicity and ischemia–reperfusion injury (FALLARINI et al., 2009).

Studies revealed that most of the natural antioxidant compounds work synergistically with each other to produce a broad spectrum of antioxidant activities that create an effective defense system against free radical attack. Synergistic effects have observed in the combinations among the rosmarinic acid, caffeic acid, carnosol and luteolin. Rosmarinic acid presented the highest capacity to repair strand breaks formation and the repair of oxidized bases (SILVA et al., 2008). Studies revealed that antioxidants like rosmarinic acid inhibits LPO and stop action of promoters with prevention of the carcinogen-DNA adduct formation (MAKINO et al., 2000; DEBERSAC et al., 2001). Effects of phytochemicals through DNA repair modulation and their interaction with other alkylating agents can be used as chemotherapeutic drugs.

The rosmarinic acid also presented anti-inflammatory properties, which are attributed to the inhibition of lipoxygenase and cyclooxygenases and interference with the complement cascade (KROL et al., 1996; PETERSEN & SIMMONDS, 2003; TICLI et al., 2005) and the inhibition of expression of inflammatory cytokines including tumor necrosis factor-a (TNF-a) interleukin (IL)-1 (GAMARO et al., 2011).

In this context the aim of the study is to evaluate the effect of rosmarinic acid on oxidative stress biomarkers and acetylcholinesterase in streptozotocin- induced diabetic rats. The findings of this study are very important for the identification of natural biologically active compound such as rosmarinic acid with possible applications in the pharmaceutical field.

2. Objectives

2.1 General Objective

The general objective of the present work was to investigate the potential role of rosmarinic acid on oxidative stress biomarkers and acetylcholinesterase in streptozotocin- induced diabetic rats.

2.2 Specific Objective

- To determine the effect of rosmarinic acid on body weight and glucose level in diabetic rats treated with rosmarinic acid.
- To analyze AChE activity in brain structures (cortex, hippocampus and striatum) in diabetic rats treated with rosmarinic acid.
- To determine the effects of rosmarinic acid in the level of lipid peroxidation in liver and kidney of diabetic rats.
- To evaluate ALA-D activity in liver and kidney of diabetic rats.
- To evaluate activity of CAT, SOD, non-protein thiol and vitamin C in liver and kidney of diabetic rats.

3. Methods and Results

All related method and results to the thesis are mentioned in the submitted manuscripts.

3.1- Chapter 1

First Manuscript

Protective effect of rosmarinic acid against oxidative stress biomarkers in liver and kidney of strepotozotocin induced diabetic rats

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- Chapter 1

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Abstract

In the present study we investigated the efficiency of rosmarinic acid (RA) in preventing alteration of oxidative parameters in liver and kidney of diabetic rat induced by streptozotocin (STZ) (55%). The animals were divided into six groups (n=8): control; ethanol; RA 10 mg/kg; diabetic; diabetic/ethanol; diabetic/RA 10mg/kg. After three weeks of treatment, we found that diabetes caused significant decreased in the activity of superoxide dismutase (SOD), catalase (CAT) and increased lipid peroxidation in liver and kidney. However, the treatment with 10 mg/kg rosmarinic acid (anitoxidant) prevented alteration in SOD and CAT activity, as well as in the levels of lipid peroxidation. In addition, rosmarinic acid reverses the decrease of vitamin C and non protein-thiol (NPSH) levels in diabetic rats. The treatment with rosmarinic acid also prevented the decrease in the Delta-aminolevulinic acid dehydratase (ALA-D) activity in liver and kidney of diabetic rats. These results indicate that rosmarinic acid effectively reduced the oxidative stress induced by STZ, suggesting that rosmarinic acid is a potential candidate in the prevention and treatment of pathological conditions in diabetic models.

***Keywords*:** Diabetes; Kidney; Liver; Rats; Rosmarinic acid.

1. Introduction

Oxidative stress plays a pivotal role in the pathogenesis of diabetes complications in both microvascular and macrovascular levels [1,2]. In a normal cell, there is an appropriate prooxidant/antioxidant balance. However, this balance can be moved towards the prooxidant when production of reactive oxygen species (ROS) is increased or when levels of antioxidants are declined [3, 4, 5]. This is called 'oxidative stress' and can result in serious cell damage.

Hyperglycemia is a link between diabetes and diabetic complications enhanced polyol activity; increased formation of advanced glycation end products; activation of protein kinase C and nuclear factor κB; and increased hexosamine pathway flux [6] which causes increased production of ROS from glucose autoxidation and protein glycosylation [7]. Inhibition of antioxidant enzymes critically affect the vulnerability of various tissues to oxidative stress and are associated with the development of complications in diabetes [8,9]. The kidney and liver are organs highly vulnerable to ROS due to the abundance of long-chain polyunsaturated fatty acids [10].

Consequence of oxidative stress in the pathogenesis of diabetes is suggested, not only by oxygen free-radical generation, but also due to non-enzymatic protein glycosylation, auto-oxidation of glucose [11], impaired glutathione metabolism [12], alteration in antioxidant enzymes [13], lipid peroxides formation and decreased vitamin C level [14].

Lipid peroxidation is associated with the oxidation of the polyunsaturated fatty acids (PUFAs) of the fatty acid membrane generates fatty acid radical [15,16]. Theses free radicals are hazardous for the viability of cells and macromolecules, such as DNA, RNA and proteins [17,18].

Enzymatic antioxidants are comprised of limited number of proteins such as catalase (CAT), glutathione peroxidase (GSH) as well as superoxide dismutase (SOD)

along with some supporting enzymes [19)] Non-enzymatic antioxidants include direct acting antioxidants, which are extremely important in defense against oxidative stress. Most of them include ascorbic and lipoic acid, polyphenols and carotenoids, derived from dietary sources [20].

δ-Aminolevulinic acid dehydratase (δ-ALA-D) has been suggested another indirect biomarker of oxidative stress [21]. δ- ALA-D enzyme catalyzes the second step in heme synthesis the condensation reaction of 2 molecules of ALA into porphobilinogen (PBG) which thus play important role in most living aerobic organisms [22], controlling the heme biosynthetic pathway. It is a metalloenzyme, containing sulfhydryl (-SH) groups and zinc, which are essential for its activity. PBG is assembled into tetra molecules which constitute prosthetic groups of physiologically relevant proteins such as hemoglobin, cytochrome, and catalase. Furthermore inhibition of this enzyme can lead to accumulation of ALA in the blood which in turn can intensify oxidative stress [23] and produce pro-oxidant effects [24] under physiological conditions [25]. Based on these results we assume that alterations in ALA-D activity could be associated with chronic oxidative stress.

In the recent years, the interest to use of medicinal plants with hypoglycemic properties in the treatment and prevention of diabetic complications has increased greatly [26]. The hypoglycemic properties of these medicinal plants for example thyme, basil, oregano are described to be due to their higher contents of antioxidants i.e. polyphenols and different bioactive compounds [27]. One of this powerful polyphenol is rosmarinic acid which was first time extracted from *Rosemarinus officinalis L*. The structure was elucidated as an ester of caffeic acid and 3-(3, 4-dihydroxyphenyl) lactic acid [28]. It is found mostly in spices and some herbs, such as: sage, lemon balm, oregano, peppermint, thyme, basil, marjoram and perilla [29]. It has many biological

properties such as inhibiting the HIV-1[30],antitumor [31], anti-hepatitis and protecting the liver, inhibiting the blood clots and anti-inflammation [32; 33]. Moreover, studies showed that rosmarinic acid is strong antioxidant than Trolox [34] and vitamin E [35]. Besides all these properties very little data available regarding hypoglycemic activity of rosmarinic acid. So, in the present study, we evaluated the effect of rosmarinic acid on, markers of oxidative stress in kidney and liver of STZ- induced rats.

2. Material and Methods

2.1 Chemicals

Rosmarinic acid was kindly gifted by Professor Nadia Mulinacci. Streptozotocin (STZ), δ-aminolevulinic acid (δ–ALA), reduced glutathione (GSH), 5,50- dithio-bis-2-nitrobenzoic acid (DTNB), thiobarbituric acid (TBA) and Coomassie brilliant blue G-250 were purchased from Sigma Chemical Co (St. Louis, MO, USA). All other reagents used in the experiments were of analytical grade and of the highest purity.

2.2 Animals

Adult male wistar rats (70-90 days; 200-250g) were used in experiment obtained from Central Animal House of the Federal University of Santa Maria, Brazil. The animals were maintained at a constant temperature (23±1°C) on a 12 h light/dark cycle with free access to food and water. Before starting the experiment, the animals were gone through an adjustment period of 20 days. All animal procedures were approved by the Animal Ethics Committee from the Federal University of Santa Maria (protocol under number: 023/2012.

2.3 Experimental induction of diabetes

Diabetes was induced by a single intraperitoneal injection of 55 mg/kg streptozotocin (STZ), diluted in 0.1 M sodium-citrate buffer (pH 4.5). The age- matched control rats received an equivalent amount of the sodium-citrate buffer. STZ-treated rats received 5% of glucose instead of water for 24 h after diabetes induction in order to reduce death due to hypoglycemic shock. Blood samples collected from the tail vein 8 days after STZ or vehicle injection. Glucose levels were measured with a portable glucometer (ADVANTAGE, Boehringer Mannheim, MO, USA). Only animals with fasting glycemia over 300 mg/dL were considered diabetic and used for the present study.

2.4 Treatment

The animals will randomly divide into six groups (8 rats per group):

1-Control

2- Ethanol

3- Rosmarinic acid 10 mg/kg body weight

4- Diabetic

5- Diabetic/ethanol

6-Diabetic/Rosmarinic acid 10 mg/kg

Two week after diabetes induction, the animals belonging to group control/rosmarinic acid 10 mg/kg and diabetic/rosmarinic acid received 10 mg/kg of rosmarinic acid, while the animals from control/saline and diabetic/saline groups received saline solution. Rosmarinic acid prepared freshly in 25% ethanol and administered via gavage, between 10 and 11 a.m. once a day during 21 days, at a volume not exceeding 0.1 mL/100 g rat weight. The choice of this dose of 10 mg/kg of

rosmarinic acid was made based on previous works that used the same concentrations of rosmarinic acid and obtained beneficial results [36,37]

In order to correct the interference of ethanol, a group of control rats and another group of diabetic rats received a solution of ethanol 25%. However, no significant statistical differences in the control/ethanol and diabetic/ ethanol groups were observed to any parameters analyzed when compared to control/saline and diabetic/saline groups, respectively (data not shown).

Twenty-four hours after the treatment, the animals previously anesthetize for blood collection by cardiac puncture and the liver and kidney removed carefully for subsequent biochemical analysis. The biological material that was not used was disposed of following biosecurity standards.

2.5. Determination of lipid peroxidation

Lipid peroxidation in liver and kidney was estimated colorimetrically by measuring thiobarbituric acid reactive substances (TBARS) using the method described previously by Ohkawa et al. [38]. In short, the reaction mixture contained 200 mL of samples of S1 from liver and kidney or standard (MDA-malondialdehyde 0.03 mM), 200 mL of 8.1% sodium dodecylsulfate (SDS), 750 mL of acetic acid solution (2.5 M HCl, pH 3.5) and 750 mL of 0.8% TBA. The mixtures were heated at 95 ^{o}C for 90 min. TBARS tissue levels were expressed as nmol MDA/mg protein.

2.6. Catalase (CAT) and superoxide dismutase (SOD) activities

For the CAT assay, liver and kidney were homogenized in 50mM potassium phosphate buffer, pH 7.5, at a proportion of 1:9 (w/v) and 1:5 (w/v), respectively. The homogenate was centrifuged at 2000 g for 10 min to yield a supernatant that was used

for the enzyme assay. CAT activity was measured by the method of Nelson & Kiesow [39]. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7), 10 mMH_2O_2 and 20 mL of the supernatant. The rate of H_2O_2 reaction was monitored at 240 nm for 2 min at room temperature. The enzymatic activity was expressed in units per mg of protein (One unit of the enzyme is considered as the amount of CAT which decomposes 1 mmol of H_2O_2 per min at pH 7 at 25 ^oC).

To perform the SOD assay [40] Kidney and liver was adequately diluted with Tris-HCl pH 7.4 at a proportion of 1:40 (w/v) and 1:60(w/v) respectively. Briefly, epinephrine undergoes auto-oxidation at pH 10.2 to produce adrenochrome, a colored product that was detected at 480 nm. The addition of samples (10, 20, 30 mL) containing SOD inhibits the auto-oxidation of epinephrine. The rate of inhibition was monitored during 180 sec. The amount of enzyme required to produce 50% inhibition was defined as 1 unit of enzyme activity.

2.7. Vitamin C and non-protein thiol group (NPSH) content

Hepatic and renal vitamin C levels were determined by the method of Jacques-Silva et al. [41]. Proteins of liver and kidney were precipitated in a cold 10% trichloroacetic acid (TCA) solution at a proportion of 1:1 (v/v) and submitted to centrifugation again. This supernatant was then used for analysis. A 300 mL aliquot of sample in a final volume of 575 mL of solution was incubated for 3 h at 37 ^oC then 500 mL H_2SO_4 65% (v/v) was added to the medium. The reaction product was determined using a color reagent containing 4.5 mg/mL dinitrophenyl hydrazine (DNPH) and CuSO4 (0.075 mg/ mL). Vitamin C levels are expressed as mg ascorbic acid/g tissue. NPSH was measured spectrophotometrically with Ellman's reagent [42] an aliquot of 100 mL for liver and 200 mL for kidney in a final volume of 900 mL of solution was

used for the reaction. The reaction product was measured at 412 nm after the addition of 10 mM 5-5-dithio-bis (2-nitrobenzoic acid) (DTNB) (0.05 mL). A standard curve using cysteine was added to calculate the content of thiol groups in samples, and was expressed as mmol SH/g tissue.

2.8.δ -Aminolevulinic acid dehydratase activity (δ-ALA-D)

Hepatic and renal δ-ALA-D activity was assayed according to the method of Sassa [43] by measuring the rate of porphobilinogen (PBG) formation, except that in all enzyme assays the final concentration of ALA was 2.2 mM. An aliquot of 200 mL of sample S1 was incubated for 0.5 h (liver) and 1 h (kidney) at 37 ^{0}C. The reaction was stopped by addition of 250 mL of trichloroacetic acid (TCA). The reaction product was determined using modified Ehrlich's reagent at 555 nm. ALA-D activity was expressed as nmolporphobilinogen (PBG) $mg^{-1}protein^{-1.}$

2.9. Protein determination

Protein was measured by the method of Bradford [44] using bovine serum albumin as standard.

2.10. Statistical analysis

Data were analyzed statistically by two-way ANOVA followed by the Duncan'smultiple tests. Differences were considered significant when the probability was $P < 0.05$.

3. Results

The body weight and blood glucose levels determined at the onset and at the end of the experiment are presented in Table 1. As can be observed, the blood glucose levels in the diabetic group treated with rosmarinic acid (10 mg/kg body weight /day) for 21 days showed no significant differences from diabetic/saline group (Table 1), while the body weight was significantly decreased in diabetic/saline group compared to normal control. Furthermore, diabetic group treated with rosmarinic acid increased the body weight compared with diabetic/saline (Table 1).

TBARS levels in liver and kidney (Fig. 1A & B) were significantly increased in the diabetic/saline group, compared to control/saline group. However, treatment with rosmarinic acid prevented an increase of lipid peroxidation in both tissues.

In the present study, decrease in the SOD activity was found both in liver and kidney (Fig. 2A & B) of STZ-induced diabetic rats compared to normal control while treatment of diabetic with rosmarinic acid (10 mg/kg body weight /day) for 21 days prevented the decrease in SOD activity in both tissues.

Similarly, CAT activity was decreased in diabetic/saline group compared with control/saline group in liver and kidney (Fig. 3A & B) while treatment of diabetic with rosmarinic acid prevented the decrease this activity.

Furthermore, in diabetic/saline group a decrease in the level of non-protein-SH was found in liver and kidney (Fig. 4A & B), compared to normal control while treatment with rosmarinic acid improved the level of non-protein-SH in diabetic group in both liver and kidney similar to control group.

We found low level of vitamin C in kidney of diabetic/saline group. However, treatment with rosmarinic acid significantly prevented the decrease in vitamin C levels (Fig. 5).

δ- ALAD activity in the liver and kidney presented a significant decrease in rats of diabetic/saline group (Fig. 6A & B). However, treatment with rosmarinic acid significantly prevented the decrease in ALA-D activity in these tissues.

These results indicate the effectiveness of rosmarinic acid in prevention of alteration in various parameters developed during oxidative stress in liver and kidney of diabetic rats.

4. Discussion

Diabetes mellitus is very common disease now-a-days both in developed and developing country and increasing day by day worldwide. There are convincing experimental and clinical studies revealed that hyperglycemia result in the formation of high levels of ROS and ultimately in the development and progression of diabetes and related complications [45,46].

Several methods have been used for induction of diabetes mellitus in animals where's STZ is commonly used for induction of experimental diabetes [47]. STZ-induced diabetes is a well-characterized experimental model of diabetes due to its ability to selectively destroy pancreatic islet of β-cells leading insulin deficiency and hyperglycemia [48].

In our study, there were significant increase in lipid peroxidation in liver and kidney of diabetic rats, as measured by TBARS formation (Fig. 1A & B). These results are in agreement with several studies that have reported an increase in TBARS levels in kidney, liver, serum and erythrocytes of animal with experimental diabetes [49,50]. In

addition, the increased lipid peroxidation under diabetic conditions could be due to increased oxidative stress in the cell as a result of the depletion of antioxidant defense systems [51]. Numerous studies showed that rosmarinic acid inhibits effectively the lipid peroxidation of cellular membranes and the protein oxidation [52]. Furthermore, RA is considered as a strong protector of oxidative stress-induced DNA damage that commonly occurs in several pathological conditions [53]. Moreover, it showed to reduce α-tocopheroxyl radical to regenerate the endogenous tocopherol, which further strengthens the antioxidant defense mechanism. The presence of CH=CH-COOH group in RA ensures greater efficiency than the COOH group found in other phenolics and this two ortho-dihydroxy groups (catechol structures) make it a stronger antioxidant and unique polyphenol unlike other [54,55].

In fact, in the present study, we found that STZ-induced diabetes decreased the level of antioxidant enzymes SOD (Fig. 2A & B) and CAT (Fig. 3A & B), as well as in NPSH levels (Fig. 4A & B) in both liver and kidney of diabetic rats.

An adequate antioxidant defense system is very necessary in a healthy body. Under normal conditions, free radicals superoxide anion (O^{2-}), the hydroxyl radical (OH-.) and hydrogen peroxide (H_2O_2) are formed in minor quantities and are rapidly scavenged by natural cellular defense mechanisms mainly enzymes like SOD and CAT and non-enzymatic antioxidants as GSH [56]. These enzymes act in two steps: firstly, SOD converts the dangerous superoxide radicals (O^{2-}) into hydrogen peroxide (H_2O_2) which is then degraded to H_2O by CAT or by glutathione peroxidase. A decrease in the activity of these antioxidants may lead to an excess of availability of $O^{2\bullet-}$ and H_2O_2, which in turn generates hydroxyl radicals, resulting in initiation and propagation of lipid peroxidation [57] and contribute to increase of oxidative stress in the diabetes mellitus [51] and consequently in the development of diabetic complications.

On the other hand, our study showed that administration of rosmarinic acid prevented the increase in TBARS levels (Fig. 1A&B) and the reduction in SOD (Fig. 2A&B) and CAT (Fig.3A&B) activity in liver and kidney of STZ-induced diabetic rats. These results are consistent with reductions in oxidative stress found in other studies, where the rosmarinic acid treatment greatly ameliorated antioxidants enzyme activities and prevented the rise in lipid peroxides in tissue and blood cells of diabetic animals [58;59]. This indicates a possible role of this flavonoid in the inactivation of free radical in diabetic state may inhibit oxidative damage of hepatic and renal tissues. The major causes for generating oxidative stress is the persisting hyperglycemia, leading to enhanced auto oxidation of glucose [60]results in the formation of hydrogen peroxide (H_2O_2) which inactivate SOD and CAT [61]. Since natural antioxidants can protect the human body from ROS and could retard the progress of many chronic diseases as well as lipid oxidative rancidity in foods [62]. Oxidation of lipids in food not only lowers the nutritional value but is also associated with cell membrane damage, and oxidative stress related diseases [63]. Therefore the addition of natural antioxidants for example rosmarinic acid to food products has become popular as a means of extending shelf life and to reduce wastage and nutritional losses by inhibiting oxidation. [64].

Since the spices like mint, oregano, basil rosemary which contain greater quantity of rosmarinic acid and other polyphenols [65] are commonly used in most countries. A standard dose of rosmarinic acid 200-300mg for oral ingestion is in common practice but there is no scientific evidence. Furthermore, there are no legal barriers to use them in food, further in vivo studies would be essential for understanding the benefits of consuming rosmarinic acid enriched herbs on human health. In present study we use comparatively less amount of rosmarinic acid (10 mg/kg) body weight in order to find out it efficiency of this dosage.

Another important aspect to be discussed in our study is that NPSH (Fig. 4 A&B) and ascorbic acid (Fig. 5) levels presented a significant decrease in kidney of diabetic/saline group compared with control. However, treatment with rosmarinic acid (10 mg/kg body weight /day) for 21 days significantly prevented the decrease in the levels of NPSH (Fig.4A & B) and vitamin C (Fig. 5) in kidney of diabetic rats. In fact, polyphenols are considered to increase the activity of γ-glutamylcysteine: the first enzyme in the glutathione biosynthesis pathway and demonstrated simultaneous escalation in the intracellular GSH level [66]. In addition, data of literature demonstrated that high levels of GSH directly detoxifies ROS and protects cellular proteins against oxidative stress through glutathione redox cycle [67, 68]. In this line, we can suggest that a prevention of a decrease in NPSH content in kidney of diabetic rats found in our study could be in part responsible for the decrease in ROS formation and in the lipid peroxidation levels and the resultant low oxidative stress obtained *in vivo* in the animals treated with rosmarinic acid.

ð-ALA-D is a sulfhydryl-containing enzyme that is extremely sensitive to oxidizing agents [21], and plays a fundamental role in most living aerobic organisms by participating in heme biosynthesis. We have previously observed that the activity of ð-ALA-D is inhibited in cases of diabetes [69]. In the present study, we observed that STZ caused a significant inhibition in the activity of ð-ALA-D in both liver and kidney (Fig. 6 A&B) and that rosmarinic acid was able to significantly relieve this inhibition. Our results are with agreement with several studies that founded a decrease in the activity of δ -ALA-D in both human and experimental diabetes. This inhibition has been related mainly to high glucose levels and overproduction of ROS [50,70].

During oxidative stress excessive accumulated aminolevulinic acid results in auto-oxidation and inhibition of δ -ALA-D may result in formation of highly reactive

cytotoxic compounds like superoxide and hydrogen peroxide which causes inflammations [71,72]. The inhibition of δ -ALA-D activity in diabetic patients is due to the oxidation of sulfhydryl groups [72,73]. δ -ALA-D is involved in the synthesis of prosthetic groups of CAT [74] and reduced activity of CAT inhibited the synthesis of ALA-D. Another factor is depletion in GSH level in diabetes which could be related to the reduction of δ-ALA-D activity as the oxidation of essential enzyme –SH groups seems to play a significant role in δ-ALA-D inhibition [73]. This shows a positive correlation between inhibited ALA-D activity and decreased NPSH levels in diabetes.

We observed that the treatment with rosmarinic acid was able to significantly relieve the inhibition of ALA-D activity in hepatic and renal tissues of diabetic rats. On the basis of these this results we can suggest that rosmarinic acid can prevent the oxidation of essential -SH groups located at its active site of δ-ALA-D and consequently its inhibition (Fig. 6 A&B). Indeed, in our study rosmarinic acid prevented the reduction of NPSH levels in hepatic and renal tissues in STZ- induced diabetic rats; hence, it could be expected to protect other endogenous thiols such as those found in δ-ALA-D enzyme. Consequently, we can suggest that the prevention of a decrease in NPSH content as well as a decrease of oxidative stress in diabetic rats by rosmarinic acid could be associated with a prevention of a decrease of δ-ALA-D activity.

In conclusion, rosmarinic acid reverses the changes in δ-ALA-D and other parameters of oxidative stress during hyperglycemic condition in liver and kidney to the level of control. Therefore, we can suggest that rosmarinic acid can be an important therapeutical agent in treatment of diabetes, contributing to prevention and reduction of oxidative damages in this endocrinopathy.

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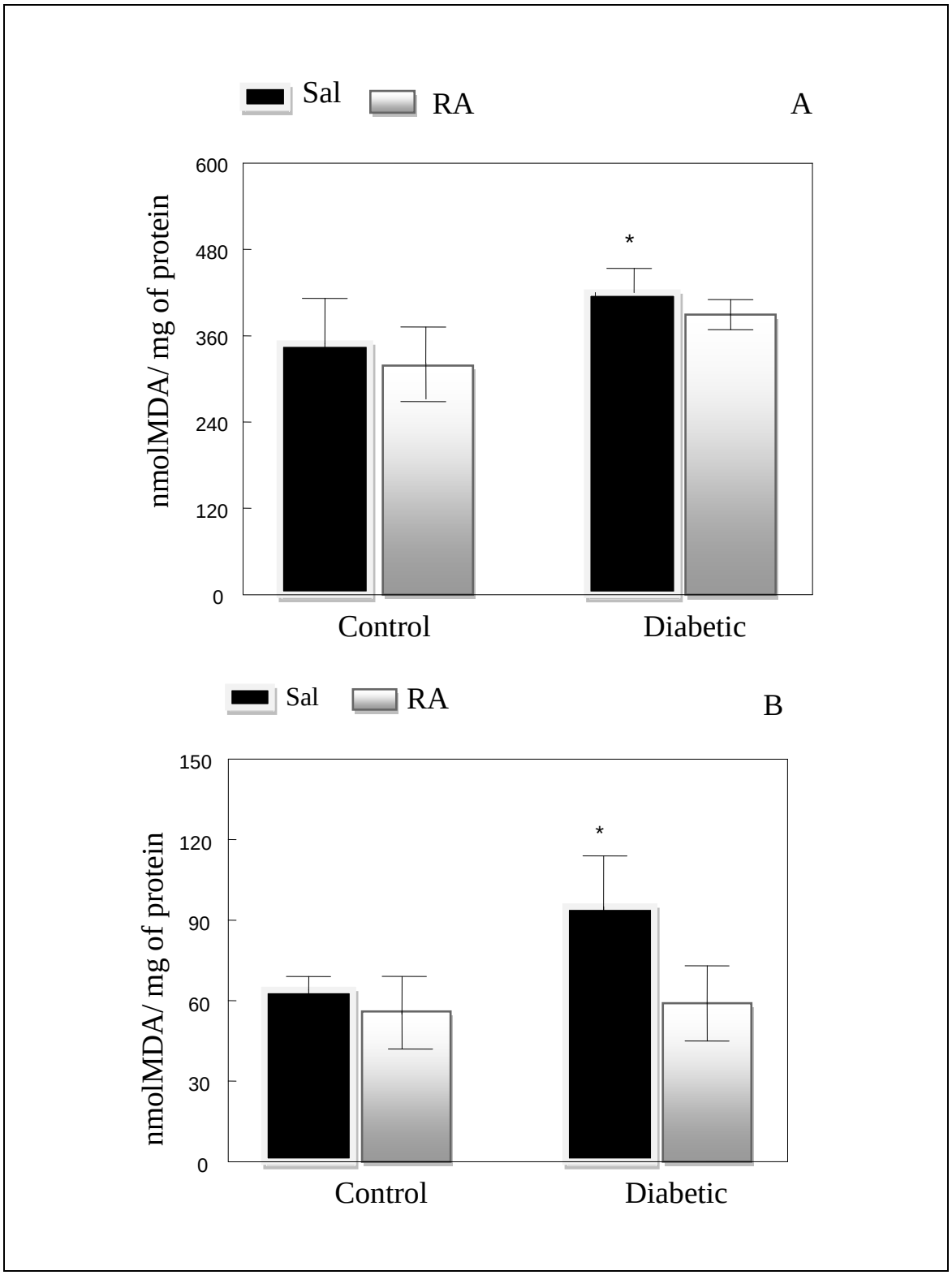

Fig.1. Levels of thiobarbituric acid reactive substances (TBARS) in liver (A) and kidney (B) of STZ-induced diabetic rats and those treated with rosmarinic acid. Bars represent means ± S.D. Groups with esteric statistically different from control ($P < 0.05$) ANOVA-Duncan's Test.

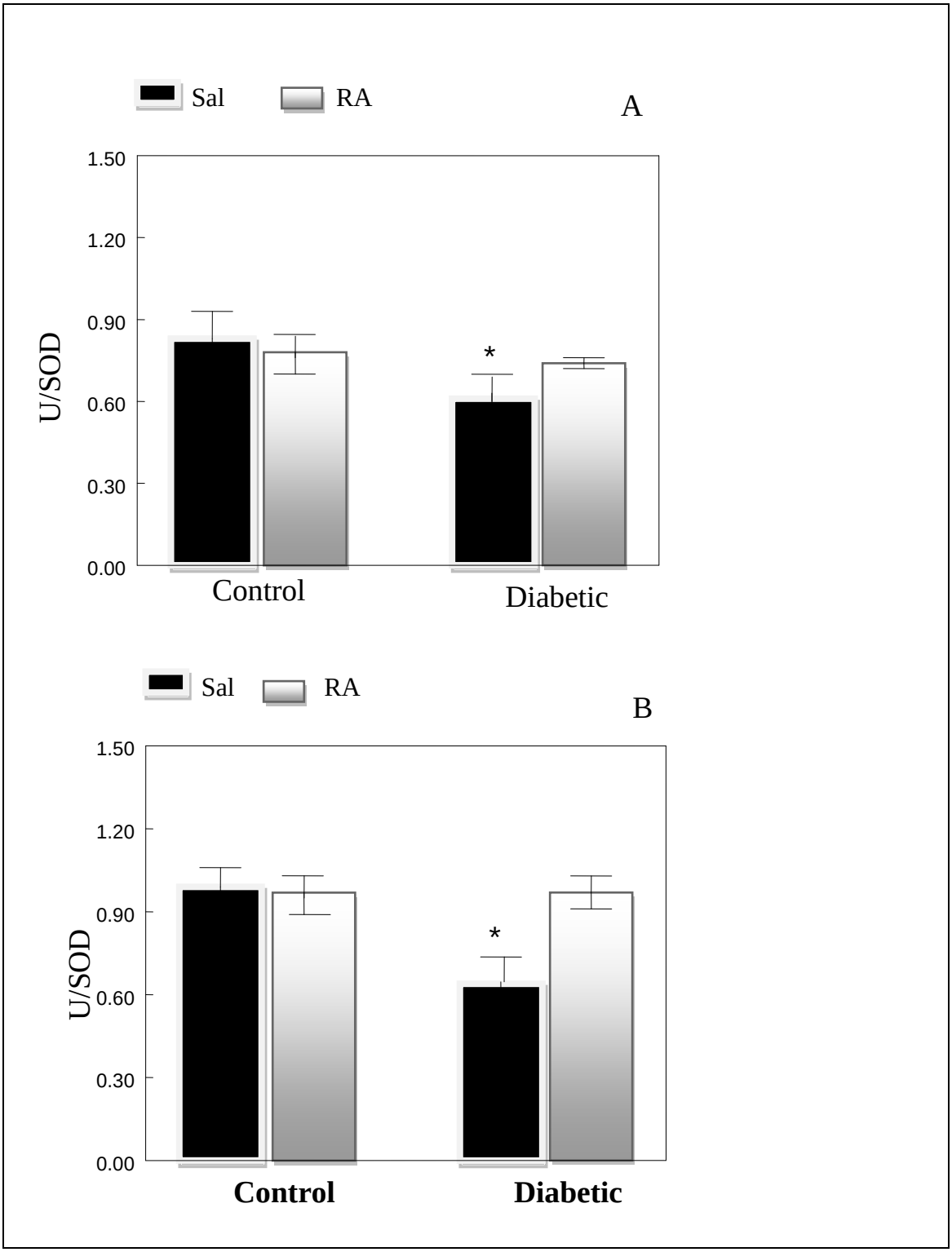

Fig. 2. SOD activity in liver (A) and kidney (B) (*P < 0.05) of STZ-induced diabetic rats and those treated with rosmarinic acid. Bars represent means ±S.D. Groups of diabetic statistically different from control. ANOVA-Duncan's Test

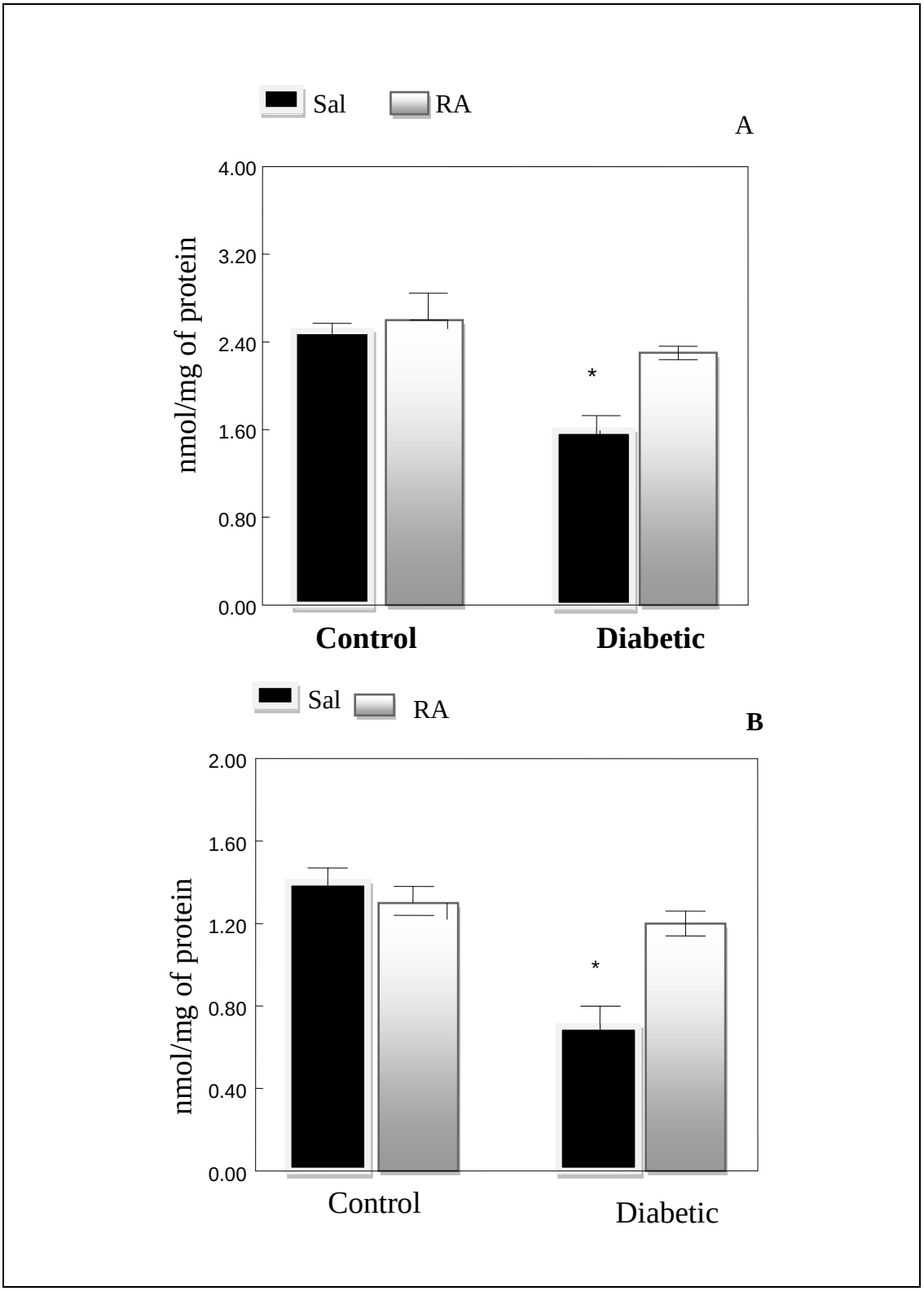

Fig.3. Catalase activity in liver (A) and kidney (B) of STZ-induced diabetic rats and those treated with rosmarinic acid. Bars represent means ±S.D. (*P < 0.05 different from control).

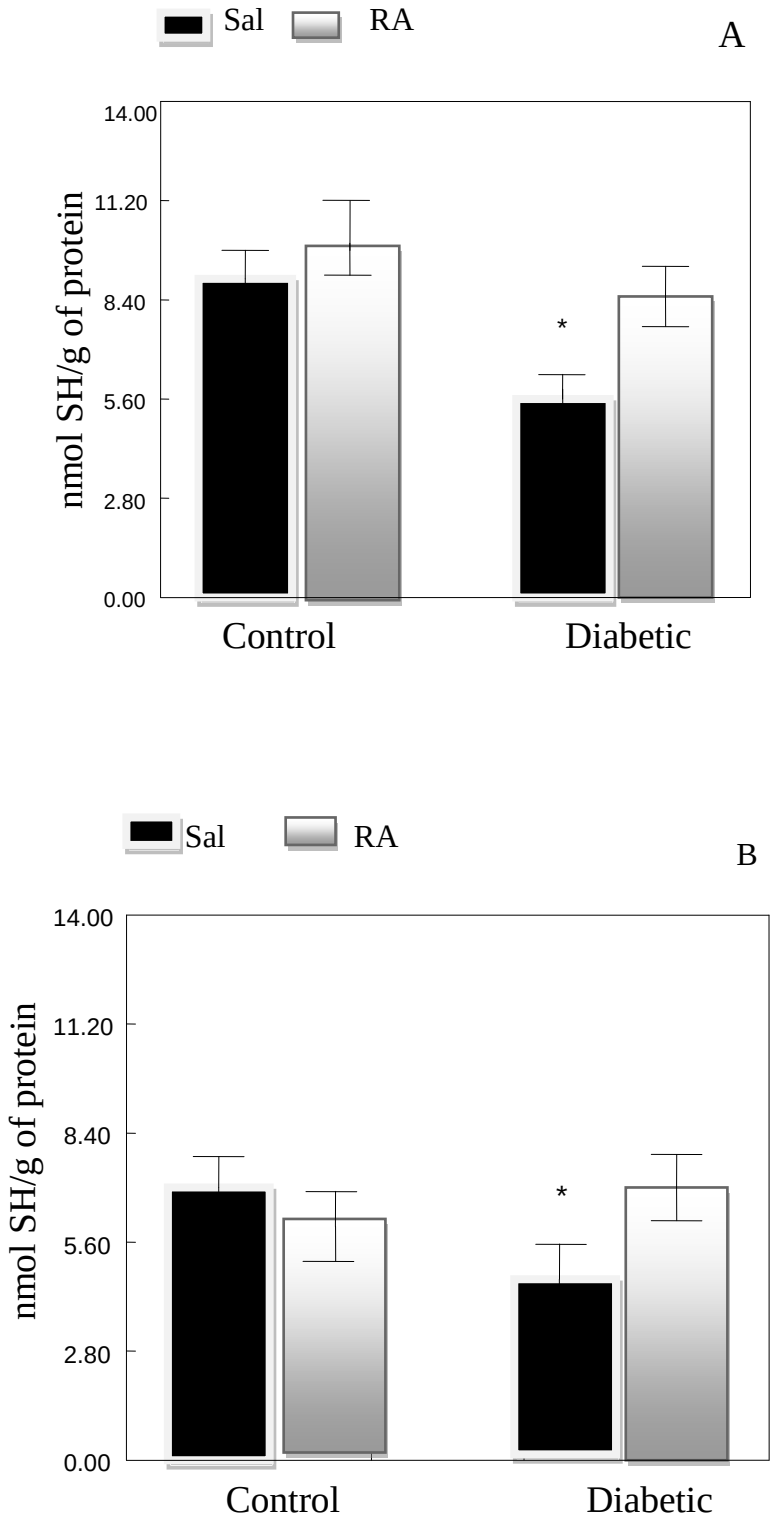

Fig. 4. NPSH activity in liver and kidney of STZ-induced diabetic rats and those treated with rosmarinicacid. Bars represent means ±S.D. (*P < 0.05). ANOVA-Duncan's Test.

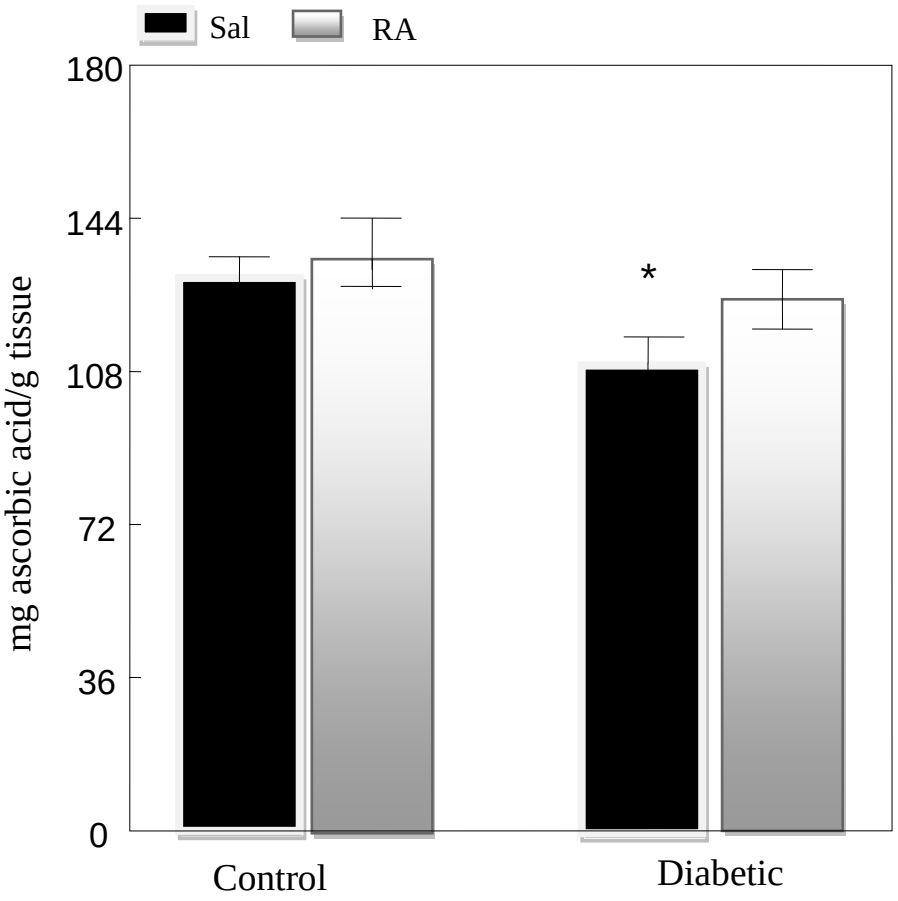

Fig. 5. Vitamin C activity in kidney of STZ-induced diabetic rats and those treated with rosmarinic acid. Bars represent means ±S.D. Groups with esteric different ($P < 0.05$). ANOVA-Duncan's Test.

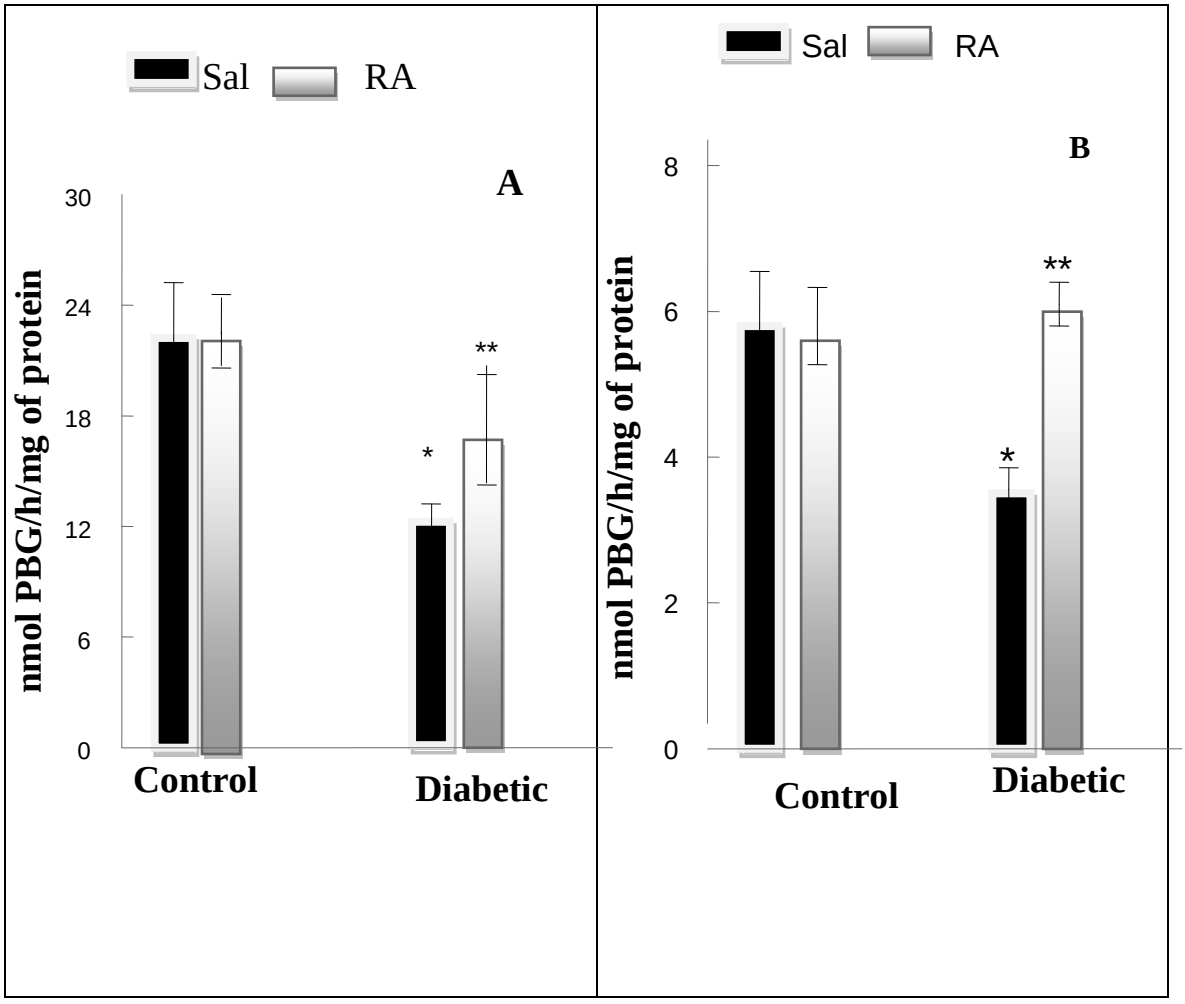

Fig.6. δ-ALA-D activity in liver (A) and kidney (B) from STZ-induced diabetic rats and those treated with rosmarinic acid. Error Bars in the graph represent means ± standard error from the eight samples per group that were tested. Significant difference from control: *P<0.05, and within diabetic**P<0.05-ANOVA-Duncan's Test.

Table 1.The effect of Rosmarinic acid (RA) after 21 days treatment on body weight and fasting blood glucose levels in control and diabetic rats at the onset and the end of the experiment

Groups	Glucose (mM)		Body weight (g)	
	Onset	End	Onset	**End**
Control/Sal	**120±10.10**	**110 ± 8.06**	**266 ± 4.50**	**284 ± 8.15**
Control/RA	**126 ± 8.86**	**132 ± 6.85**	**267 ± 5.19**	**299 ± 9.40**
Diabetic /Sal	**460 ± 20.28**	**478 ± 19.32**	**250 ± 5.09**	**189 ±15.44***
Diabetic/ RA	**502 ± 32.13**	**502 ± 23.56**	**200 ± 4.17**	**262±7.47***

3.2- Chapter 2

2nd Manuscript

Rosmarinic acid prevents lipid peroxidation and increase in acetylcholinesterase activity in brain of streptozotocin-induced diabetic rats

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Abstract

We investigated the efficacy of rosmarinic acid (RA) in preventing lipid peroxidation and increased activity of acetylcholinesterase (AChE) in the brain of streptozotocin (55%) (STZ)-induced diabetic rats. The animals were divided into six groups (n=8): control; ethanol; RA 10 mg/kg; diabetic; diabetic/ethanol; diabetic/RA 10 mg/kg. After 21 days of treatment with rosmarinic acid the cerebral structures (striatum, cortex and hippocampus) were removed for experimental assays. The results demonstrated that low dose of rosmarinic acid (10 mg/kg) significantly reduced the level of lipid peroxidation in hippocampus (28%), cortex (38%) and striatum (47%) of diabetic rats. In addition, it was found that hyperglycemia caused significant increased in the activity of AChE in hippocampus (58%), cortex (46%), and striatum (30%), where rosmarinic acid reversed this effect or maintained the level of control after three week treatment. The results showed that rosmarinic acid can be used to overcome lipid peroxidation and central nervous system (CNS) complication through inhibition of AChE. We suggest that rosmarinic acid can be used as a therapeutic agent for the treatment of diabetes.

***Keyword**:* Streptozotocin; diabetes; lipid peroxidation; acetylcholinesterase; rosmarinic acid.

1. Introduction

Diabetes mellitus, a major crippling disease refers to the group of diseases that lead to high blood glucose levels resulting from either low levels of the hormone (insulin) or from abnormal resistance to insulin's effects.[1] The prevalence of diabetes for all age-groups worldwide was estimated to be 2.8% in 2000 and 4.4% in 2030.[2] During diabetes persistent hyperglycemia causes increased production of free radicals, as a result of glucose auto-oxidation and protein glycosylation.[3,4] High level of lipid peroxidation has been found in diabetic patients. Peroxidation of membrane lipids seriously impair membrane functions and disturb ionic gradient receptor and transport functions, results in cellular dysfunctions.[5,6] In addition, increased thiobarbituric acid reactive substances (TBARS) in rats with STZ-induced diabetes is a well-established method for monitoring lipid peroxidation.[7]

It has been observed that reactive oxygen species (ROS) contribute to the development of chronic complications in the CNS.[8,9] Furthermore, several studies reveal that neuronal death is a common feature of diabetes and Alzheimer's disease.[10,11]

Acetylcholinesterase (AChE 3.1.1.7) is a membrane bound enzyme that hydrolyzes neurotransmitter acetylcholine (ACh) into choline and acetate after their function in cholinergic synapses at the brain region.[12] The AChE is present in higher amount in healthy human brain compare to other tissues of the body.[13]Abnormalities affecting AChE activity have been reported in various diseases including diabetes.[14,15]

Literature reveals the role of antioxidants and suggests that there is strong association between high intake of antioxidants and low incidence of diseases linked with free radicals like diabetes.[16,17] It has been proved that plants are source of

compounds with antioxidant properties.[18] This activity is mostly related to phenolic compounds such as rosmarinic acid.[19] It is a well-known natural product found in rosemary (*Rosmarinus officinalis*),lemon balm (*Melissa officinalis*), and other medicinal plants like thyme, oregano, savory, peppermint, sage.[20,21,22] Interestingly, previous studies of our research group also demonstrated that polyphenols, such as resveratrol, prevent the increase in AChE as well the increase in lipid peroxidation.[23,24] However, the effects of rosmarinic acid in these parameters still were not determined. Thus, the principal aim of the present study was to evaluate anti acetylcholinesterase property of rosmarinic acids in hyperglycemia and its protective role against lipid peroxidation in STZ-induced diabetic rats.

2. Materials and Methods

2.1 Chemicals

Coomassie brilliant blue G-250; 5,5′dithiobis-2-nitrobenzoic acid (DTNB), acetylthiocholine iodide, Rosmarinic acid kindly gifted by Nadia Mulinacci from Italy. Streptozotocin was obtained from Sigma Chemical Co (St. Louis, MO, USA). All other reagents used in experiments were of analytical grade.

2.2 Animals

Adult male wistar rats (70-90 days; 200-250g) were used in experiment obtained from Central Animal House of the Federal University of Santa Maria, Brazil. The animals were maintained at a constant temperature (23±1°C) on a 12 h light/dark cycle with free access to food and water. Before starting the experiment, the animals were

gone through an adjustment period of 20 days. All animal procedures were approved by the Animal Ethics Committee from the Federal University of Santa Maria (protocol under number: 023/2012

2.3 Experimental induction of diabetes

Diabetes was induced by a single intra-peritoneal injection of 55 mg/kg STZ, diluted in 0.1 M sodium-citrate buffer (pH 4.5). The age- matched control rats received an equivalent amount of the sodium-citrate buffer. STZ-treated rats received 5% of glucose instead of water for 24 h after diabetes induction in order to reduce death due to hypoglycaemic shock. Blood samples collected from the tail vein 8 days after STZ induction. Glucose levels were measured with a portable glucometer (ADVANTAGE, Boehringer Mannheim, MO, USA). Only animals with fasting glycaemia over 300 mg/dl were considered diabetic and used for the present study.

2.4 Treatment

The animals were randomly divided into six groups (8 rats per group):

1-Control;

2- Ethanol;

3- Rosmarinic acid 10 mg/kg;

4- Diabetic;

5- Diabetic/ethanol

6- Diabetic/Rosmarinic acid 10 mg/kg.

Two weeks after diabetes induction, the animals belong to the group control/ rosmarinic acid and diabetic/rosmarinic acid received 10 mg/kg body weight of rosmarinic acid, while the animals from control and diabetic/ groups received saline solution. Rosmarinic acid prepared freshly in 25% ethanol and administered via gavage, between 10 and 11 a.m. once a day during 21 days, at a volume not exceeding 0.1 ml/100 g rat weight. The choice of this dose of 10 mg/kg of rosmarinic acid was made based on previous works that used the same concentrations of rosmarinic acid and obtained beneficial results.[25,26]

Rosmarinic acid was dissolved in 25% ethanol. In order to correct the interference of ethanol, a group of control rats and another group of diabetic rats received a solution of ethanol 25%. However, no significant differences in the control/ethanol and diabetic/ ethanol groups were observed to any parameters analyzed when compared to control/saline and diabetic/saline groups, respectively (data not shown).

Twenty-four hours after the last treatment, the animals were previously anesthetized for blood collection by cardiac puncture and the liver, kidney and brain removed carefully for subsequent biochemical analysis.

2.5. Brain tissue preparation

The animals were submitted to euthanasia being previously anesthetized halothane and brain structures were removed and separated into cortex, hippocampus, and striatum placed in a solution of 10 m M Tris–HCl, pH 7.4, on ice. The brain structures were homogenized in a glass potter in Tris-HCl solution. Aliquots of resulting brain structure homogenates were stored at −8°C until utilization. Protein was

determined in each structure: cerebral cortex (0.7 mg/ml), striatum (0.4 mg/ml), hippocampus (0.8 mg/ml).

2.6 Protein Determination

Protein in different structure of rat's brain was determined by method of Bradford et al. (1976)[27] using bovine serum albumin as a standard solution.

2.7 Determination of lipid peroxidation

Lipid peroxidation in brain hippocampus, striatum and cortex was determined according to Ohkawa et al. (1979).[28] The amount of thiobarbituric acid reactive substances (TBARS) was expressed as nmol MDA/ mg tissue.

2.8. Determination of AChE activity

The AChE enzymatic assay was determined by a modification of the spectrophotometric method of Ellmann et al. (1961)[29] as previously described by Rocha et al. (1993).[30] The reaction mixture (2 ml final volume) contained 100 mM K+-phosphate buffer, pH 7.5 and 1 mM 5,5′-dithiobisnitrobenzoic acid (DTNB). The method is based on the formation of the yellow anion, 5,5′-dithio-bis-acid-nitrobenzoic, measured by absorbance at 412 nm during 2-min incubation at 25°C. The enzyme (40–50 μg of protein) was pre-incubated for 2 min. The reaction was initiated by adding 0.8 mM acetylthiocholine iodide (AcSCh). All samples were run in duplicate or triplicate and the enzyme activity were expressed in μmol AcSCh/h/mg of protein.

2.9. Statistical analysis

Experimental data analysed by using analysis of variance two ways ANOVA followed by an appropriate post hoc test.

3. Results

The body weight and blood glucose levels determined at the onset and at the end of the experiment are presented in Table 1. As can be observed, the blood glucose levels in the diabetic group treated with rosmarinic acid (10 mg/kg body weight /day) for 21 days showed no significant differences from diabetic group (Table 1), while the body weight was significantly decreased in diabetic group compared to control. Furthermore, diabetic group treated with rosmarinic acid increased the body weight compared with diabetic (Table 1).

In diabetes, this brain region also become affected and showed increased level of lipid peroxidation in hippocampus (Figure 2), cortex (Figure 3) and striatum (Figure 4) when compared with control. The increased lipid peroxidation was decreased or maintained to the level of control by rosmarinic acid in hippocampus (28%), cortex (38%) and striatum (47%) of diabetic rats (Figure 2-4).

In the present study, increased AChE activity level was found in hippocampus (58%) (Figure 5) of STZ-induced diabetic rats compared to control (Figure 5). In addition, cortex (46%) (Figure 6) and striatum (30%) (Figure 7) also showed high level of AChE activity in diabetic group compared to normal control. In parallel experiments diabetic rats treated with rosmarinic acid (10 mg/kg body weight /day) given by gavage

for a period of 3 weeks, decreased the activity of AChE in hippocampus, cortex and striatum, (Figure 5-7) compared to diabetic/saline group.

4. Discussion

Hyperglycemia is the main reason of causing a series of biochemical events which result in the formation of high levels of ROS and ultimately an oxidative stress.[31] STZ-induced diabetes is a well characterized experimental model for type 1 diabetes due to its ability to selectively destroy pancreatic islet of β-cells leading insulin deficiency and hyperglycemia.[32] In STZ-induced diabetic rats a decreased body weight was observed (Table 1). There are different views about this loss of weight for example it may related to excessive break-down of tissue proteins,[33] or dehydration and catabolism of fats and proteins.[34]

Free radicals react with important biological molecules (nucleic acids, proteins and lipids etc). However, the most vulnerable ones are polyunsaturated fatty acids. Reaction of free radicals with cell membrane constituents leads to lipid peroxidation.[35] In our study an increased of lipid peroxidation in hippocampus (Figure 2), cortex (Figure 3) and striatum (Figure 4) was observed in diabetic rats as evidenced by increase in TBARS levels. This increased in lipid peroxidation levels during the diabetes can be due to inefficient anti-oxidant system.[36] In fact, several studies have demonstrated a decrease in antioxidant enzymes, such as SOD and CAT and consequently in increase TBARS levels, in brain of diabetic rats, which can contribute to oxidative damages in central nervous system.[37,38] and consequently results in development and progression of several neurodegenerative disease. Furthermore, it is reported that high level of lipid peroxidation is responsible for the formation of lipid

hydroperoxides in membrane, which result in damage of membrane structure and alteration of membrane-bound enzymes like AChE.[39]

In the present study, we found significant high activity of AChE in hippocampus (Figure 5), cortex (Figure 6) and striatum (Figure 7) of STZ- induced diabetic rats compared with normal control group. Similarly, SCHMATZ et al., (2009) and SANCHEZ-CHAVEZ & SALCEDA, (2000) also observed a significant elevation in AChE activity in cerebral cortex, striatum and hippocampus of STZ -induced diabetic rats. Interestingly, AChE activation leads to a fast ACh degradation and a subsequent downstimulation of ACh receptors causing undesirable effects on cognitive functions.[41]

In this context, we can suggest that the increase in AChE activity caused by experimental diabetes leads to a reduction in the efficiency of cholinergic neurotransmission due to a decrease in acetylcholine levels in the synaptic cleft, thus contributing to the progressive cognitive impairment and other neurological dysfunctions seen in diabetic patients.[42] On the other hand, Ach is considered an anti-inflammatory molecule, and a possible reduction in the levels due to increase of AChE activity found in our study, can contribute to increase the levels of IL-1 and TNF-α due to the absence of the negative feedback control exerted by this neurotransmitter. All these events can lead to enhance local and systemic inflammation.[12,43] In fact, Diabetes Mellitus and Alzheimer diseases share a common feature of low-grade systemic inflammatory conditions in which plasma AChE activity is increased.[44]

Treatment of diabetes mellitus and its complications in the recent context have focused on the usage of naturally occurring antioxidants in food or medicinal flora to replace synthetic antioxidants, which are being restricted, due to their adverse side effects, such as carcinogenicity .[45,46] Several studies had shown that plants are source of compounds with antioxidant property and prevent lipid peroxidation in various tissues

during induced oxidative stress. The activities are mostly related to phenolic compounds.[24, 47,48]

In the present study, rosmarinic acid (RA) (Figure1), decreased lipid peroxidation in hippocampus (Figure 2), cortex (Figure 3) and striatum (Figure 4) of diabetic rats. These results are in according with other studies that have showed the effects antioxidant of rosmarinic acid, reducing the levels of MDA in central nervous system.[49]

An important aspect to be discussed in our study is that the prevention of increase of TBARS levels by rosmarinic acid can be associated with the anticholinesterase property exhibit by this polyphenol. In fact, the treatment with rosmarinic acid prevented the increase in AChE activity of hippocampus (Figure 5), cortex (Figure 6) and striatum (Figure 7) of diabetic rats after 21 days treatment. These results are similar to those found in studies with other antioxidants polyphenols that also prevented the rise in AChE activity. This effect in AChE enzyme can contribute to increase the ACh levels in the synaptic cleft enabling an improvement in cognitive functions, such as learning and memory[50], which suggests an interaction between rosmarinic acid and the cholinergic system. On the other hand, it is important to point out that that the effects protects against oxidative stress, decreasing lipid peroxidation in brain of diabetic rats observed in the treatment with rosmarinic acid could be a decisive factor to the prevention of alteration in AChE activity. In fact, alterations in the lipid membrane observed during the diabetic state can be directly associated with modification of the conformational state of the AChE molecule and would explain change activity of this enzyme in diabetic state.[51]

In conclusion, the results obtained in the present study demonstrate an increase in lipid peroxidation in brain from diabetic rats that were associated with alterations in AChE activity indicating that cholinergic neurotransmission is altered in the diabetic state. In addition, the treatment with rosmarinic acid prevented the increase in AChE activity and of lipid peroxidation, demonstrating that this compound may modulate cholinergic neurotransmission and may consequently improve cognitive dysfunctions associated to oxidative stress. Thus, we can suggest that rosmarinic acid is a promising natural compound with important neuroprotective actions which should be investigated in future studies in order to find a better therapy for patients with cholinergic disorders caused by the hyperglycemic state.

Acknowledgements

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Legend of the Figures

Figure.1. Chemical structure of rosmarinic acid.

Figure 2. Protective role of rosmarinic acid in STZ- induced diabetic rats via inhibition of lipid peroxidation in hippocampus. Rosmarinic acid was given by gavage for three weeks at the rate of 10 mg/kg body weight. The result represents the mean of eight different experiments of each group down in duplicate. * $P < 0.05$, diabetic group show significant difference from all groups

Figure 3. Lipid peroxidation in STZ-induced diabetic rats in cortex and those treated with rosmarinic acid (10 mg/kg) after three weeks. The results represent the means of 8 different experiments down in duplicate. * $P < 0.009$, show significant difference from all groups.

Figure 4. Rosmarinic acid decreases the level of lipid peroxidation in rat striatum after 21 days treatment at 10 mg/kg. The diabetic groups indicate significant (* $P<0.0009$) difference from all groups. The results represent the mean of eight different experiments of each group down in duplicate.

Figure 5. In hippocampus, AChE activity levels in STZ-induced diabetic rat model and treated with rosmarinic acid (mean ± SD, n = 8). Significant differences from other groups (*$p \leq 0.05$).

Figure 6. Acetylcholinesterase activity in cortex of STZ-induced diabetic rats and those treated with rosmarinic acid (10 mg/kg body weight) after three weeks treatment. Bars represent means ±S.E.M. * $P<0.001$, significant increase compare to other groups.

Figure 7. Acetylcholinesterase activity in striatum of STZ-induced diabetic rats and those treated with rosmarinic acid (10 mg/kg body weight) after three weeks treatment. Bars represent means ±S.E.M. * $P<0.05$, significant increase compare to other groups.

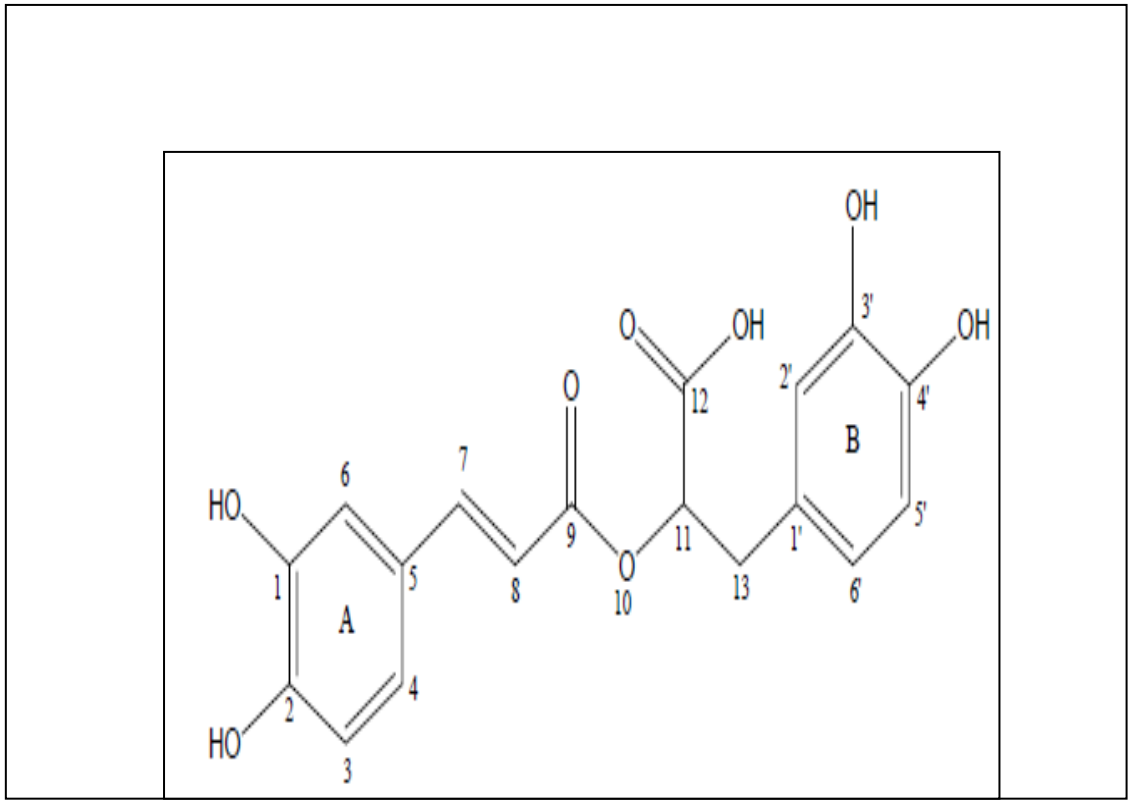

Figure.1 Rosmarinic acid

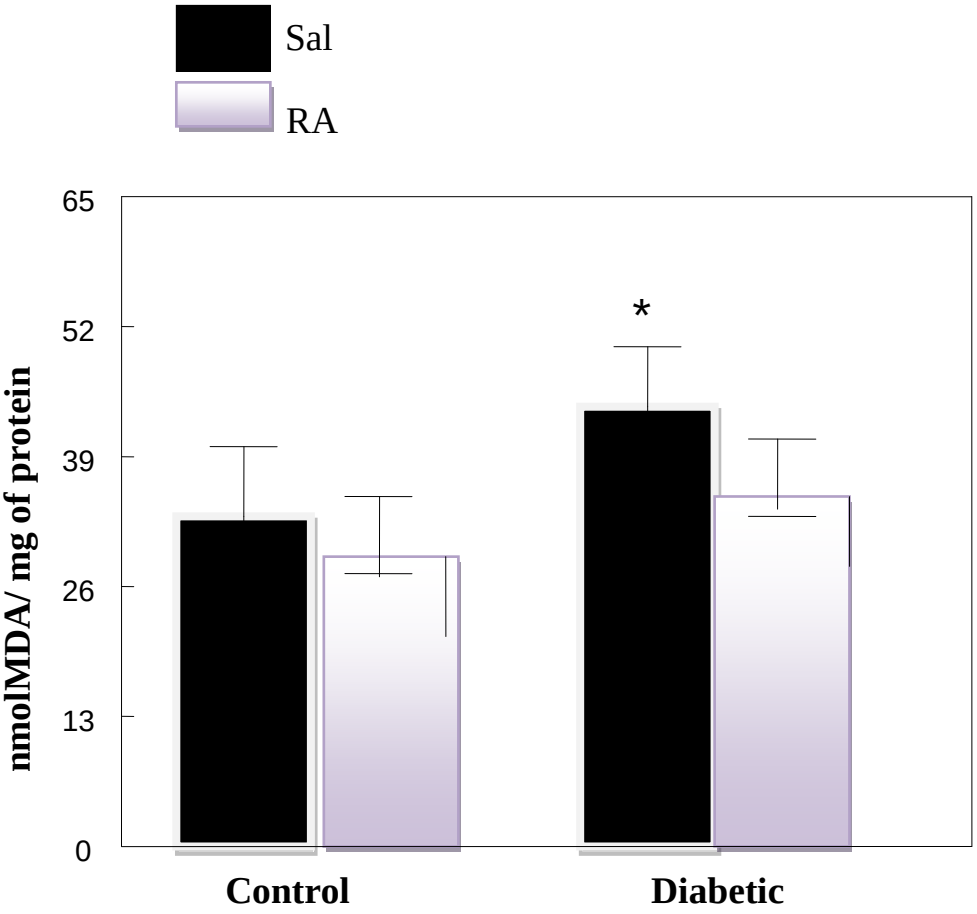

Figure. 2 Hippocampus

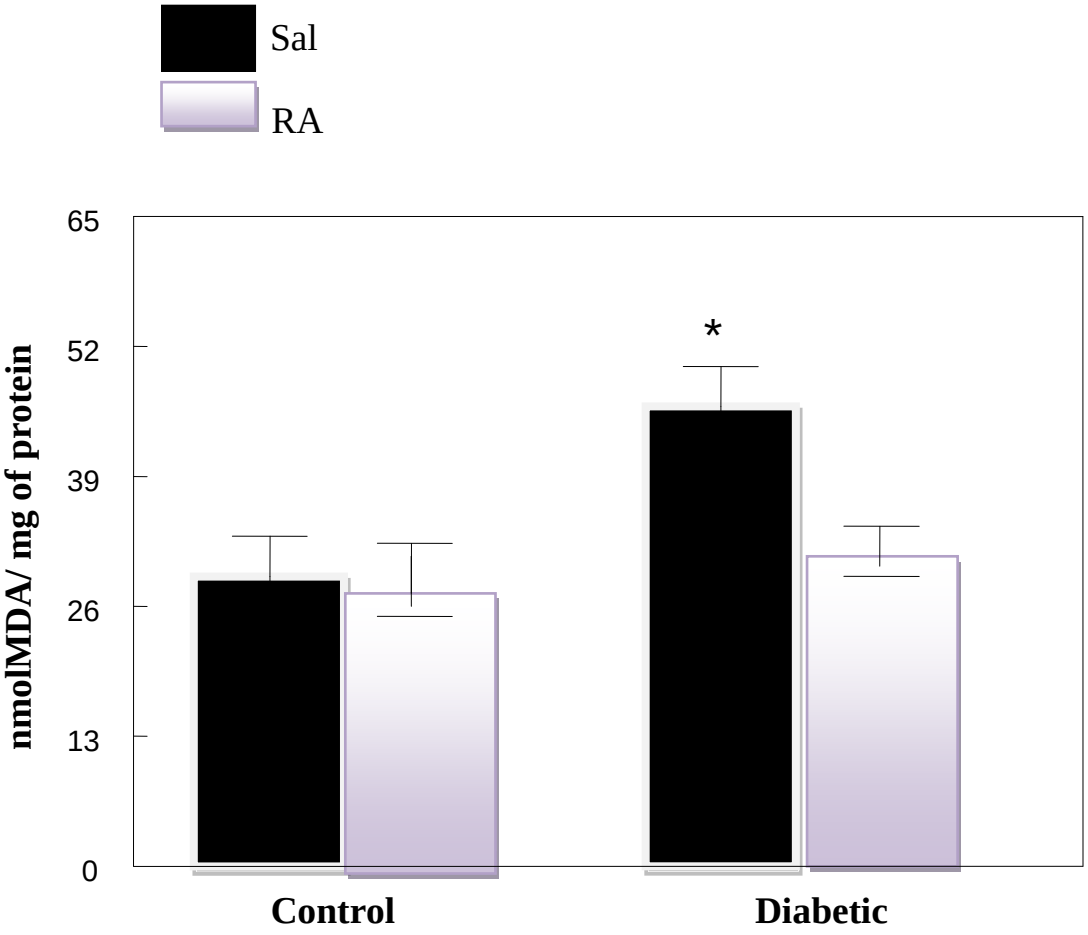

Figure. 3 Cortex

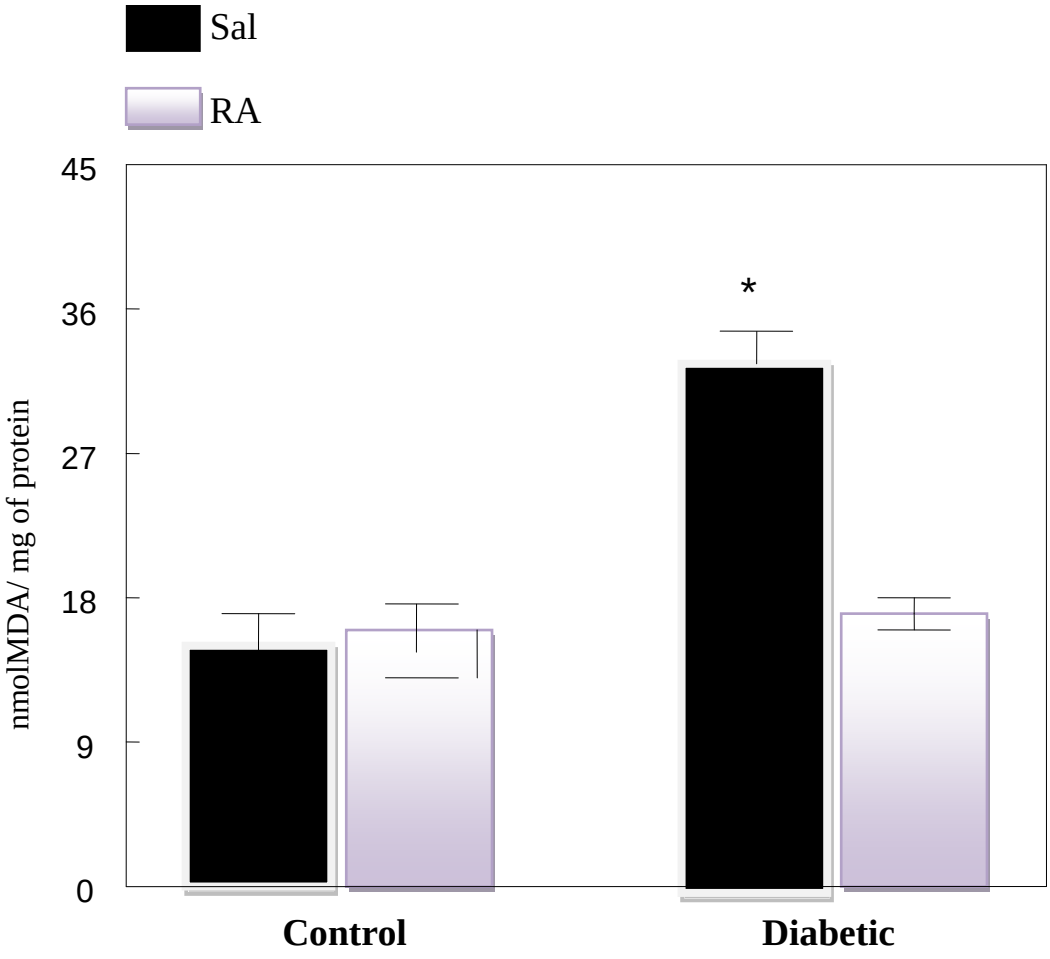

Figure. 4 Striatum

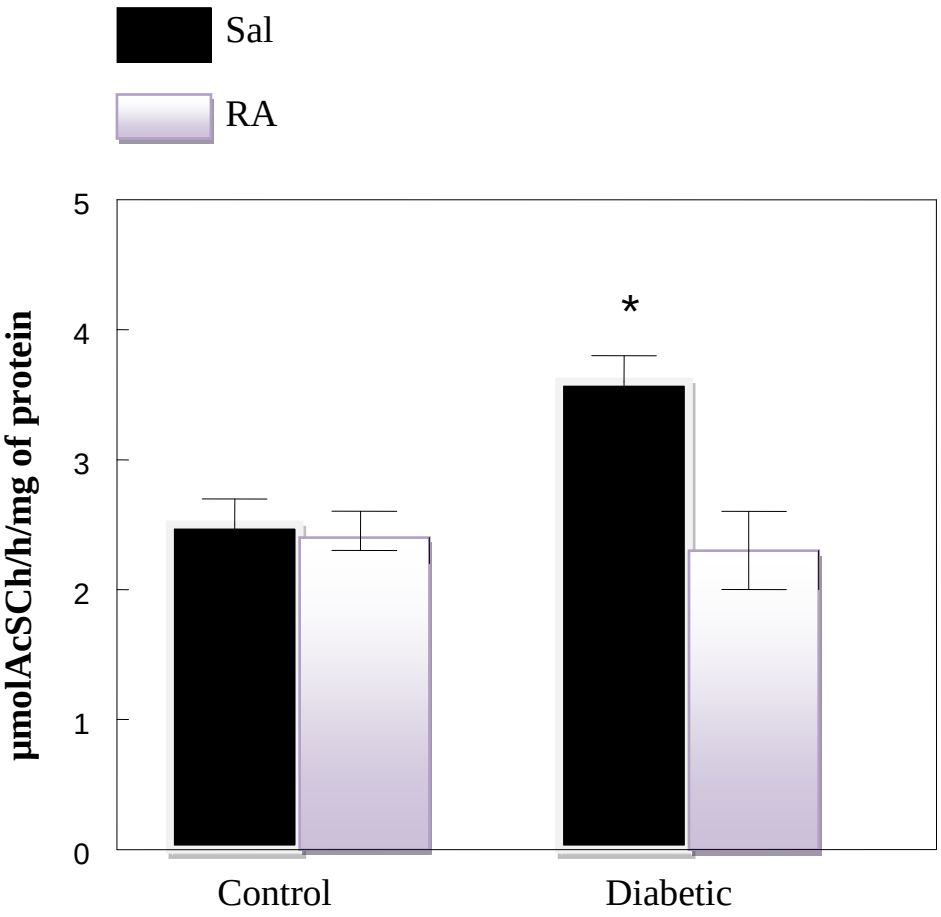

Figure. 5 Hippocampus

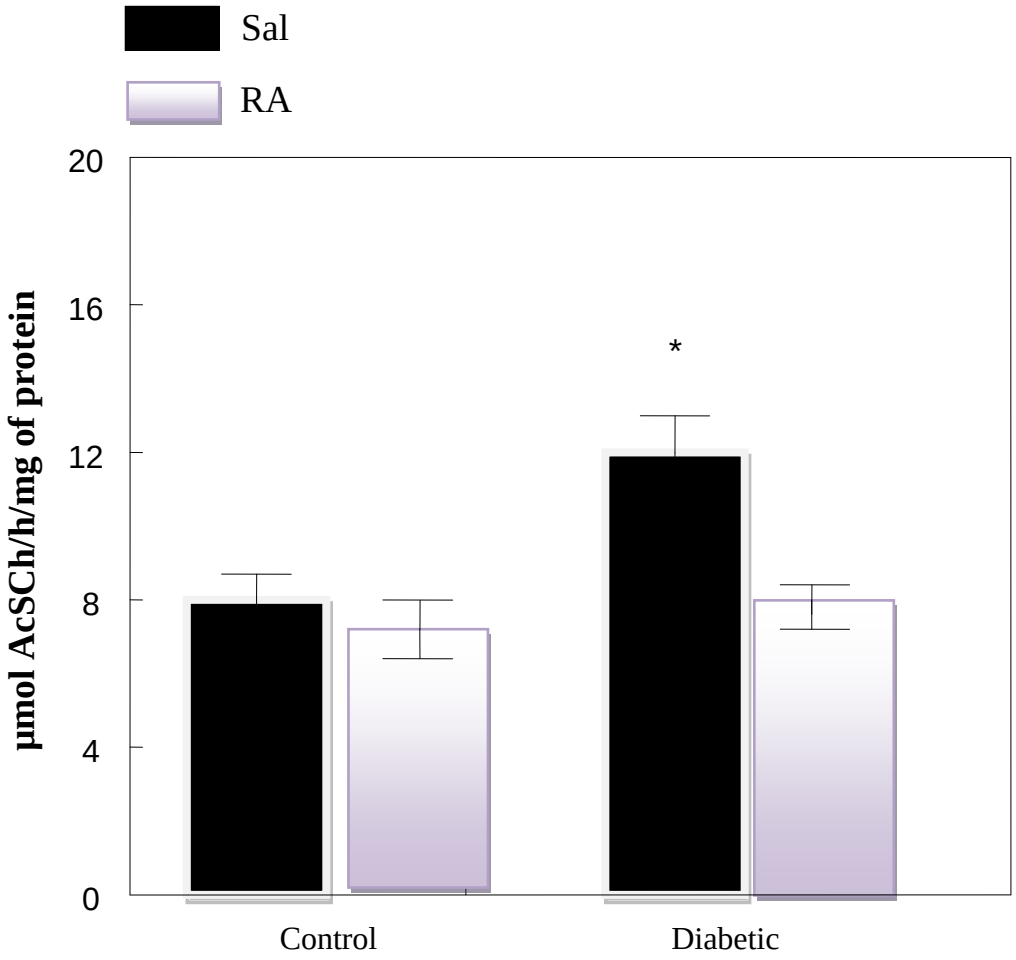

Figure. 6 Cortex

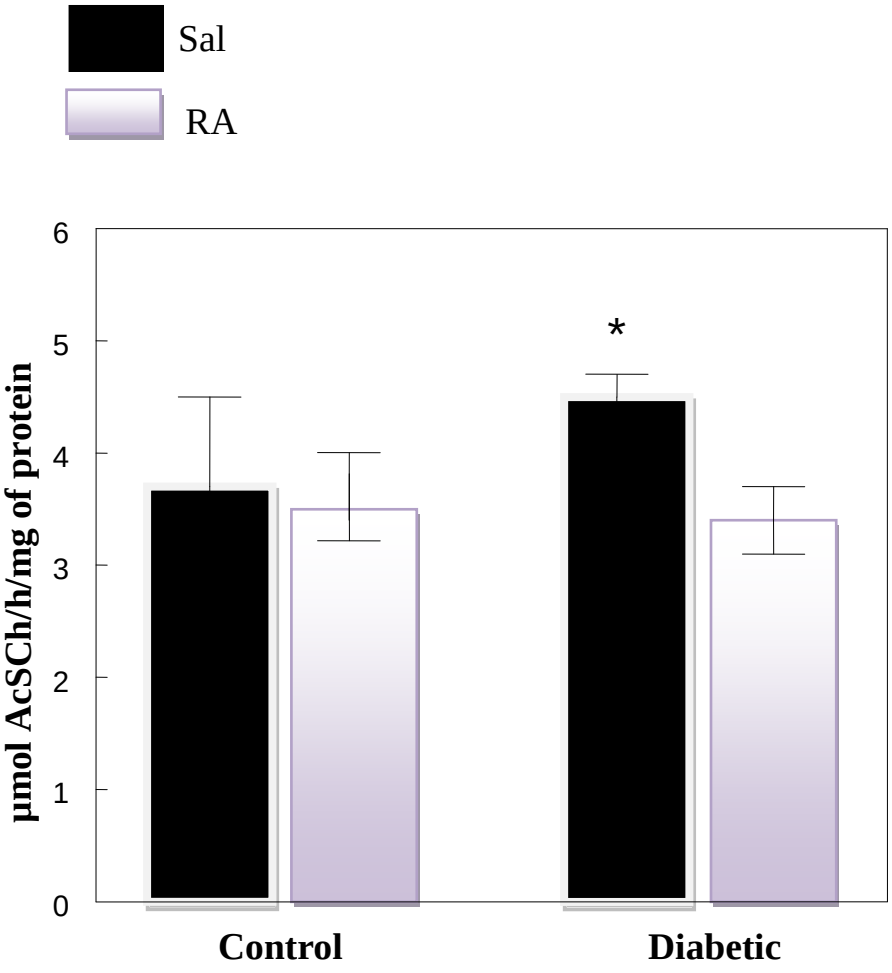

Figure.7 Striatum

Table 1.The effect of rosmarinic acid (RA) after 21 days treatment on body weight and fasting blood glucose levels in control and diabetic rats at the onset and the end of the experiment.

Groups	Glucose (mM)		Body weight (g)	
	Onset	End	Onset	**End**
Control	**120±10.10**	**110±8.06**	**266 ± 4.50**	**284 ± 8.15**
RA	**126 ± 8.86**	**132 ± 6.85**	**267 ± 5.19**	**299 ± 9.40**
Diabetic	**460± 20.28**	**478± 19.32**	**250 ± 5.09**	**189 ±15.44***
Diabetic/RA	**502± 32.13**	**502± 23.56**	**200 ± 4.17**	**262 ± 7.47***

4. Discussion

Various epidemiological studies have repeatedly revealed an inverse correlation between the risk of chronic human diseases and the consumption of polyphenolic compound rich diet (CHECKOWAY et al., 2002; SCALBERT et al., 2005; KURIYAMA et al., 2006; BENETOU et al., 2008). Medicinal plants having antioxidants compounds become an interesting tool for the treatment of diabetes complications (KAVISHANKAR et al., 2011). These medicinal plants possess anti-diabetic effects due to presence of the bioactive agents for example alkaloids, glycosides, galactomannan gum, polysaccharides, hypoglycans, peptidoglycans, guanidine, steroids, glycopeptides and terpenoids (MENTREDDY, 2007). Many ethnobotanical surveys on medicinal plants used by the local population have been performed in different parts of the world (MASIKA & AFOLAYAN, 2003; ERASTO et al., 2005; KODURU et al., 2007; COOPOOSAMY & NAIDOO, 2011). The treatment of diabetes with synthetic drugs is costly and having side effects. Therefore, medicinal plants are an alternative source for the treatment of diabetes (PANDAY et al., 2012). As these plants exhibit valuable antioxidant properties, mostly related to phenolic compounds such as rosmarinic acid (KUMAR et al., 2010).

Rosmarinic acid (RA)), an ester of caffeic acid and 3,4-dihydroxyphenyllactic acids, is a phenolic compounds present in rosemary (*Rosmarinus officinalis*), lemon balm (*Melissa officinalis*), and other the medicinal plants like thyme, oregano, savory, peppermint, sage (ZHENG & WANG, 2001). It has been found that *Rosmarinus officinalis,* the main source of rosmarinic acid, was able to inhibit or reversed oxidative stress parameters in the STZ- induced diabetic rats (KHALIL et al., 2012).

The results of our study show a significant increase in lipid peroxidation in liver and kidney of diabetic rats. These results are in agreement with several others who reported an increase in the level of TBARS in tissues of diabetic animal models (SANCHEZ-CHAVEZ & SALCEDA, 2000; SATHISHSEKAR & SUBRAMANIAN, 2005). Several studies indicated that diabetes mellitus is accompanied by an increase in free radicals and a reduction in antioxidant activity (MOSAAD et al., 2004;BASHAN et al., 2009). Thus, the balance between free radical formation and the defense system is impaired. This imbalance causes damage to cell components including proteins, lipids and nucleic acids (RAHIMI et al., 2005). This damage leads to lipid peroxidation that form lipid peroxides, which decompose to form numerous products including MDA (PILZ et al., 200; HUANG & ZHENG, 2006) after rupture of the carbon chain of unsaturated fatty acids (SUTTNAR et al., 2001). These products have been known to cross-link membrane components and result in altered membrane permeability, lipid organization and cellular dysfunction (ACWORTH et al., 1997; EVANS et al. 2002; WRIGHT et al., 2006). In the present study decrease in CAT, SOD activities and GSH levels could be due to increase in the lipid peroxidation product, malondialdehyde, which can cross-link with amino group of protein to form intra and intermolecular cross-links thereby inactivating several enzymes.

The administration of rosmarinic acid in different tissues prevented the decrease in the activities of the antioxidant enzymes suggesting their role in improving antioxidant system in diabetes. One of the important roles of antioxidants is to inhibit the chain reaction of lipid peroxidation (PITIPANAPONG et al., 2007). They react with free radicals, which are the main promoters of the auto-oxidation of fatty acid chain of fat, thereby terminating the chain reaction and limiting free radical cellular damage (GÜLÇIN et al., 2004; ELMASTAS et al., 2007).

This study also observed a decrease in the levels of non-protein thiol (NPSH) and vitamin C in the liver and kidney of diabetic rats. This decrease in the levels of vitamin C could be due to the increased utilization of vitamin C in deactivation of the increased levels of ROS or to decrease in the GSH level, since, the GSH is required for the recycling of vitamin C (LI et al., 2001). Administration of rosmarinic acid prevented the decrease in the level of vitamin C in liver and kidney of diabetic rats, may be expected to enhance the GSH levels or stimulation of the system to recycle the dehydro ascorbic acid back to ascorbic acid.

Once oxidized, glutathione can be reduced back by glutathione reductase, using NADPH as an electron donor (TANDOĞAN& ULUSU, 2006). The reduced availability of NADPH may be due to reduced synthesis or increased metabolization of NADPH through some other pathway, could be also responsible for low levels of reduced glutathione in STZ- diabetic rats as compared to control rats (MADHU et al., 1996). Administration of rosmarinic acid restores the decreased level of NPSH in liver and kidney of diabetic rats.

Glucose utilization is decreased in the brain during diabetes (MCCALL, 1992; AHMED & ZAHRA, 2011), providing a potential mechanism for increased vulnerability to acute pathological events. It is well recognized that altered membrane functions in several tissues including brain occur due to an increase free radicals which results in increased lipid peroxidation of the cellular membranes (HALLIWELL & GUTTERIDGE, 2001). Our results showed increased level of lipid peroxidation in hippocampus, cortex and striatum in diabetes rat when compared with control. However the treatment with rosmarinic acid (10 mg/kg) significantly reduced the level of lipid peroxidation in hippocampus (28%), cortex (38%) and striatum (47%) of

diabetic rats, reinforcing the antioxidant role of this polyphenol in the prevention of oxidative damage.

Prolonged exposure to chronic hyperglycemia in diabetes can lead to various complications, including neurological disorders (BROWNLEE, 2001). Acetylcholine is the primary neurotransmitter of the cholinergic system and its activity is regulated by AChE enzyme (SILMAN & SUSSMAN, 2005). The termination of nerve impulse transmission is accomplished through the degradation of acetylcholine into choline and acetate by AChE (WEIHUA et al., 2000). Thus, AChE activity has been used as a marker for cholinergic activity (ELLMAN et al., 1961). It has been well established; that alterations in the lipid membrane observed during the diabetic state can be directly associated with modification of the conformational state of the AChE molecule and would explain the change activity of this enzyme in diabetic state (SANCHEZ-CHAVEZ et al., 2005). In the present study, we found significant high activity of AChE activity in hippocampus (58%), cortex (46%), and striatum (30%), where rosmarinic acid reversed this effect or maintained the level of control in STZ- induced diabetic rats compared with normal control group.

It is important to note that this high AChE activity in hippocampus can be attributed to damage to presynaptic and postsynaptic structures, dysregulation of Ca2+ homeostasis, neuronal loss, dendritic atrophy in CA3 neurons, reduced expression of insulin growth factors and their receptors and decreased neurogenesis (JACKSON-GUILFORD et al., 2000; SARAVIA et al., 2004). All these marked pathological changes effect the brain of diabetic animals, particularly the hippocampus.

We also evaluated effect of rosmarinic acid on δ-ALA-D enzyme. In the present study, δ-ALA-D activity was inhibited in the liver and kidney of diabetic rats. Our data are in accordance with SCHMATZ et al. (2011) & SOUZA, et al., (2007). Inhibition of

the enzyme leads to disturbances of heme biosynthesis and results in intermediate accumulation, which has been shown to induce pro-oxidant events (KELADA et al., 2000; ROCHA et al., 2004). Different factors contribute to this inhibition of δ-ALA-D activity. High glucose concentration inhibit the enzyme activity by two distinct mechanisms: by involving the oxidation of cysteinyl residues and by glycation of the active site lysine residue involved in Schiff's base formation with the first δ-ALA molecule (FOLMER et al., 2003, 2004). Subsequently, this Schiff's base adduct is converted to stable glycation products Amadori. This process generates ROS from the glycated proteins under physiologic conditions (JAMES et al., 2011). Excessive formation of these glycation products appears to be the common biochemical link between chronic hyperglycemia and development of long-term diabetic complications (that affect the eyes, kidneys, and nervous system) (CHEVALIER et al., 2002).

Moreover SH group of δ-ALA-D when oxidized by free radicals or after formation of adducts with reactive chemicals impairs its enzymatic activity. The impair activity is also linked to the significant reduction in the antioxidant system especially in NPSH, which is responsible for preventing the oxidation of the sulphydryl groups (BONFANTI et al., 2012). In line with this, in the present study, the activity of δ-ALA-D was significantly decreased in hepatic and renal tissues of diabetic rats. From the results obtained, we can suggest that the treatment with rosmarinic acid could be associated with the prevention of decrease δ-ALA-D activity by the decrease in NPSH content as well as decrease of oxidative stress in diabetic rats.

So in these lines the findings of the present study demonstrated (Figure. 8) that rosmarinic acid treatment may provide effective protection against oxidative damage in liver, kidney and brain of STZ- induced diabetic rats, since this compound was able to ameliorate enzymatic and non-enzymatic antioxidant defense system and to prevent the

lipid peroxidation in these tissues. In addition treatment with rosmarinic acid was able to prevent the increase in AChE activity in cerebral structures of diabetic rats, demonstrating that this compound can modulate cholinergic neurotransmission and consequently improves cognition. Taken together, these results may contribute to a better understanding of the protective role of rosmarinic acid, emphasizing the influence of this polyphenol and other antioxidants in the diet for human health, possibly preventing hepatic, renal and neuronal complications associated with diabetes mellitus.

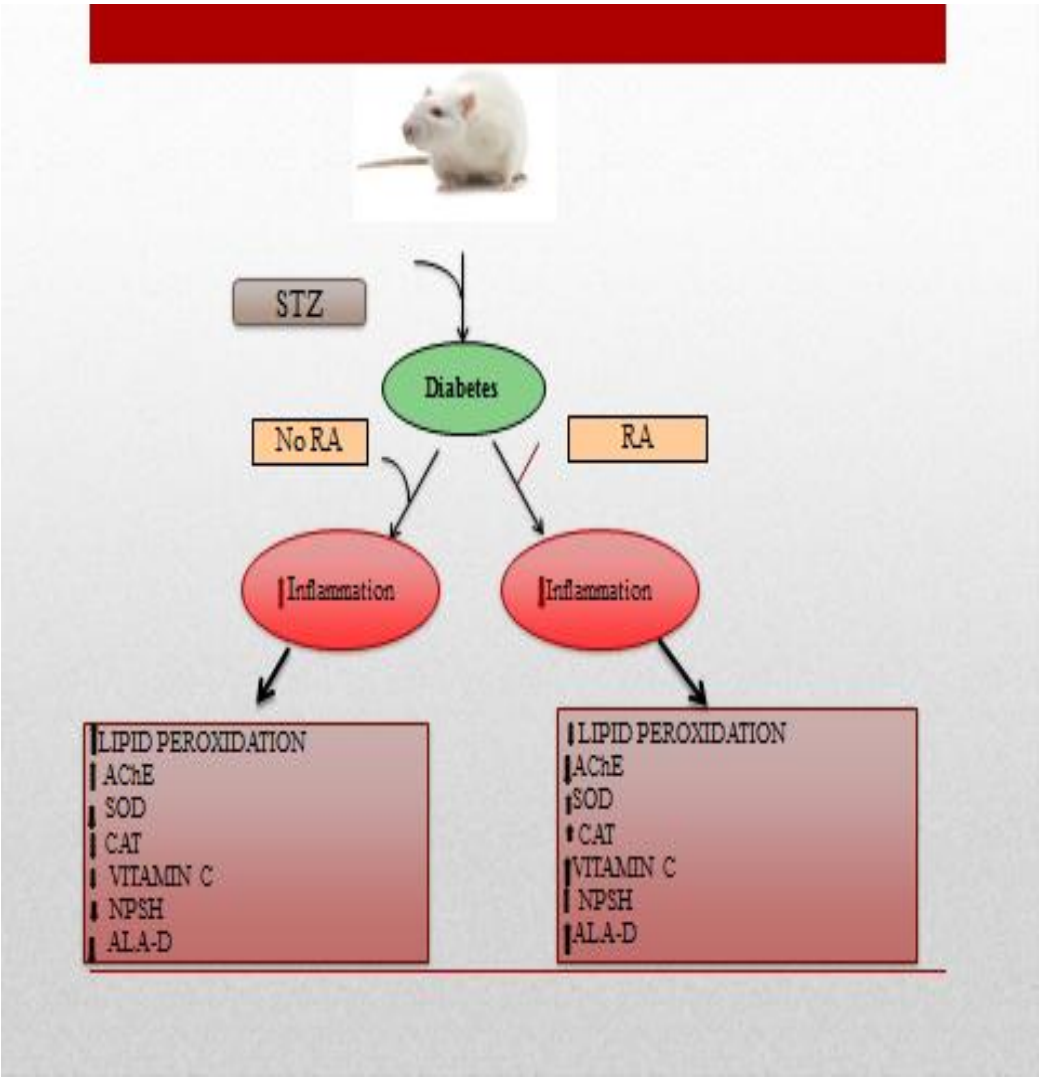

Figure. 8. Schematic representation of the study**.**

5. Conclusions

- Diabetes mellitus has been shown to be a state of increased oxidative stress with decrease in the activity of SOD, CAT and the levels of vitamin C and NPSH accompanied by increased levels of TBARS in liver and kidney of diabetic rats.
- Rosmarinic acid treatment may provide effective protection against oxidative damage in liver and kidney of STZ- induced diabetic rats, since this compound was able to ameliorate enzymatic and non-enzymatic antioxidant defense system. The activity of δ-ALA-D inhibited in liver and kidney in diabetic rats. Treatment with rosmarinic acid prevented this inhibition, emphasizing the importance of antioxidant compounds to minimize the deleterious effects of diabetes on the activity of this important enzyme.
- The increase in lipid peroxidation in brain from diabetic rats associated with alterations in AChE activity indicating that cholinergic neurotransmission is altered in the diabetic state. Treatment with rosmarinic acid prevented the increase in AChE activity and of lipid peroxidation, demonstrating that this compound may modulate cholinergic neurotransmission and may consequently improve cognitive dysfunctions associated to oxidative stress.

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