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Combined Therapy of Rutin and Silymarin has More Protective Effects on Streptozotocin-Induced Oxidative Stress in Rats

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ABSTRACT

The present study has designed to evaluate the combined effect of rutin (RT) and silymarin (SM) on oxidative stress in STZ-induced diabetic rats. Diabetic rats exhibited increased serum glucose, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), acid phosphatase (ACP), triglyceride (TG), total cholesterol (TC), creatinine (Cr), urea (BUN) and C-reactive proteins with significant decrease in serum insulin, total protein (TP) and albumin levels. Pro-inflammatory biomarkers including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-1β (IL-1β) levels significantly increased in diabetic rats. The activities of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST) and the levels of reduced glutathione (GSH) were decreased while increases in the levels of thiobarbituric acid reactive substances (TBARS) were observed in liver. Oral treatment with RT or/and SM to diabetic rats for a period of 6 weeks showed significant ameliorative effects on all the biochemical parameters studied. These results indicated that combined supplementation of RT and SM has more potential ameliorative effects on diabetic-induced oxidative stress compared to their individual effect; hence the combined therapy could be more effective than an adjuvant therapy for the prevention and/or management of diabetes and aggravated antioxidant status.

INTRODUCTION

Diabetes mellitus (DM), a life threatening as well as life style modifying metabolic disorder is characterized by hyperglycemia or diminished insulin secretion, or both. Symptoms of DM include polyuria, polydipsia, weight loss, polyphagia and blurred vision (Van Zandt *et al.*, 2004). According to the International Diabetes Federation (IDF), there were 151 million diabetic patients in 2000. This number reached to 194 million in 2003 and to 221 million in 2010 and is expected to be 334 million in 2025 (Mehuys *et al.*, 2008). Prevalence of DM has tremendously increased in Saudi population from last two decades. In a recent epidemiology study, Alqurashi et al. (2011) reported the prevalence of DM among the Saudi population is about 30% and by hoping it may go up to 50% in next decade. Hyperglycemia leads to numerous acute & chronic complications most of them are associated with increased lipid peroxidation (LPO), which may contribute to long term tissue damage (Saravanan and Ponmurugan, 2011). Various studies have shown that DM is associated with oxidative stress, leading to an increased production of reactive oxygen species (ROS), including superoxide radical, hydrogen peroxide (H_2O_2), and hydroxyl radical or reduction of antioxidant defense system (Peerapatdit *et al.*, 2006; Sklavos *et al.*, 2010). ROS are involved in the pathogenesis of many diseases including hypoxia, hypercholesterolemia, atherosclerosis, hypertension, ischemia reperfusion injury and heart failure (Wilcox and Gutterman, 2005).

STZ has been widely used for inducing diabetes in the experimental animals through its toxic effects on pancreatic b-cells, possibly through generating excess ROS (Kim *et al.*, 2003). The cytotoxic action of STZ is also associated with the generation of ROS causing oxidative damage (Lenzen, 2008). LPO is a key marker of oxidative stress and it increased as measured by indices of elevated LPO byproducts TBARS, depletion of endogenous antioxidant, and antioxidant enzymes activities in plasma and tissues are commonly found in rats with STZ-induced diabetes, and

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these alterations may cause tissues to be more susceptible to oxidative damage (Nizamutdinova *et al.,* 2009; Likidlilid 2010). Antioxidant refers to a compound that can inhibit the oxidation of lipids by deterring the initiation of oxidative chain reactions and which can thus prevent or repair damage done to the cells by oxygen. These compounds act by one or more of the following mechanisms: reducing activity, free radical-scavenging, potential complexing of pro-oxidant metals and quenching of singlet oxygen. The formation of ROS is prevented by an antioxidant system that included non-enzymatic antioxidants (vitamin C, vitamin D and glutathione), enzymes regenerating the reduced forms of antioxidants, and ROS-scavenging enzymes such as SOD, CAT, GPx and GST (Adewole and Ojewole, 2008; Budin *et al.,* 2009). Epidemiological studies have shown that many phytonutrients of fruits and vegetables might protect the human body against damage by ROS. The consumption of natural antioxidant phytochemicals was reported to have potential health benefits (Rangkadilok *et al.*, 2007). Flavonoids are known to have powerful antioxidant activity that could play a protective role in oxidative stress-mediated diseases, and recent attention has focused on the potential uses of flavonoids-based drugs for the prevention and treatment of these pathologies (Kamalakkannan and Prince, 2006; Lopez-Revuelta *et al.,* 2006; Mirshekar *et al.*, 2010; Lu *et al.,* 2010). RT and SM are flavonoids having strong antioxidant properties. Several experimental studies showed their potential effects individually against oxidative stress induced by metabolic disorders including diabetes, inflammation, hepatotoxicity and cardiovascular diseases (Lopez-Revuelta *et al.*, 2006; Choi et al. 2006; Nencini *et al.,* 2007; Soto *et al.,* 2003). When multiple antioxidants are used in combination, they protect against vulnerability to other agents and synergistically potentiate their antioxidant properties (Aleisa *et al.,* 2013). These synergistically potentiated antioxidant effects of agents contribute to the improvement of cognitive function. Thus the present study was designed to investigate the additive hepatoprotective effects of RT and SM combination against diabetic-induced oxidative injury following STZ injection to male Wistar rats.

MATERIALS AND METHODS

Animals

Wistar male albino rats, roughly the same age of 8-10 weeks and weighing 250-280 g were received from the Experimental Animal Care Center (College of Pharmacy, King Saud University, Riyadh, Saudi Arabia). They were maintained under controlled conditions of temperature (22±1ºC), humidity (50-55%) and light (12 h light/dark cycles) and were provided with Purina chow (Grain Silos & Flour Mills Organization, Riyadh, Saudi Arabia) and water *ad libitum*. All procedures including anesthesia procedure were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, Institute for Laboratory Animal Research (NIH Publications No. 80-23; 1996, USA) and the Ethical Guidelines of the Experimental Animal Care Center (College of Pharmacy, King Saud University, Riyadh, Saudi Arabia). Pre-experimental procedure the ethical clearance has taken from Experimental Animal Care Center.

Diabetes induction

Diabetes was induced in overnight fasted rats by a single intraperitoneal injection of STZ (SIGMA Chemicals, USA) at a dose of 65 mg/kg body weight freshly dissolved in 0.1 mol/L citrate buffer, pH 4.5. Control rats as vehicle received equal volume of citrate buffer. The animals with fasting blood glucose values more than 250 mg/dl after 72 h of STZ injection were considered diabetic and included in the study.

Study design

Six normal healthy rats were used in control group (vehicle) and the STZ-induced diabetic rats were randomly divided as, diabetic (STZ), RT (100mg/kg/day) treated to diabetic rats (RT100+STZ), SM (60mg/kg/day) treated to diabetic rats (SM60+STZ) and RT+SM (50 and 30 mg/kg/day respectively) treated to diabetic rats (RT50+SM30+STZ). Vehicle and drug treatment were started three weeks after the diabetes induction and continued for six consecutive weeks. Weekly body weight of each rat was recorded and the general health and behavior of animals in each group were monitored during the entire study. Finally, animals were sacrificed under ether anesthesia, blood samples were obtained by cardiac puncture and left for 30 min to coagulate then centrifuged at 4000 rpm for 10 min. Serum samples were separated and stored at -70˚C till analysis. Whole liver, kidneys and spleen were dissected from each rat, weighed and calculated its ratio with body weight (g/100 g body weight) then part of liver was preserved at -70 ˚C till analysis.

Serum Biochemical parameters

In serum, glucose, AST, ALT, ALP, ACP, TP, CRP, albumin, TG, TC, Cr and urea (BUN) levels were estimated by using commercially available kits (RANDOX Laboratories Ltd., Diamond Road, Crumlin, Co., Antrim UK) and insulin levels were measured by insulin enzyme immunoassay (ELISA) kit (DRG, Germany). Serum pro-inflammatory cytokines including TNF-α, IL-6 and IL-1β concentrations were assayed by an enzyme-linked immunosorbent assay kit (ShangHai SenXiong Science and Technology Company, China). The levels were estimated by following the instruction provided by the manufacturer.

Liver tissue preparation

Liver samples were homogenized in 50 mM phosphate buffered saline (pH 7.4) by using a glass homogenizer (Omni International, Kennesaw, GA, USA). Half of the homogenates were centrifuged at 1000 g for 10 min at 4ºC to separate nuclei and unbroken cells. The pellet was discarded and a portion of supernatant was again centrifuged at 12000 g for 20 min to obtain post-mitochondrial supernatant. In homogenate, TBARS and levels were estimated. In post-mitochondrial supernatant, SOD, CAT, GST and GPx activities were measured.

Estimation of TBARS levels in liver

A TBARS assay kit (ZeptoMetrix) was used to measure the lipid peroxidation products, malondialdehyde (MDA) equivalents. One hundred microliters of homogenate was mixed with 2.5 ml reaction buffer (provided by the kit) and heated at 95 °C for 60 min. After the mixture had cooled, the absorbance of the supernatant was measured at 532 nm using a spectrophotometer. The lipid peroxidation products are expressed in terms of nmoles MDA/mg protein using molar extinction coefficient of MDA-thiobarbituric chromophore (1.56 x 10^5/M/cm).

Estimations GSH levels in liver

The levels GSH were measured using the method described by Sedlak and Lindsay, 1968. Homogenate was mixed with 0.2 M Tris buffer, pH 8.2 and 0.1 mL of 0.01 M Ellman's reagent, [5,5'-dithiobis-(2-nitro-benzoic acid)] (DTNB). Each sample tube was centrifuged at 3000 g at room temperature for 15 min. The absorbance of the clear supernatants was measured using spectrophotometer at 412 nm in one centimeter quarts cells.

Estimations of SOD activity in liver

The activity of SOD in liver was estimated using the method described by Kono, (Kono, 1978) with the aid of nitrobluetetrazolium as the indicator. Superoxide anions are generated by the oxidation of hydroxylamine hydrochloride. The reduction of nitrobluetetrazolium to blue formazon mediated by superoxide anions was measured 560 nm under aerobic conditions. Addition of superoxide dismutase inhibits the reduction of nitrobluetetrazolium and the extent of inhibition is taken as a measure of enzyme activity. The SOD activity was expressed as units/mg protein.

Estimation of CAT activity in liver

The CAT activity was measured by the method of Aebi, (Aebi, 1974.) using hydrogen peroxide as substrate in post-mitochondrial supernatant. The hydrogen peroxide decomposition by catalase was monitored spectrophotometrically (LKB-Pharmacia, Mark II, Ireland) by following the decrease in absorbance at 240 nm. The activity of enzyme was expressed as units of decomposed/min/mg proteins by using molar extinction coefficient of H_2O_2 (71/M/cm).

Estimations of GST activity in liver

The GST activity in liver was measured by the method of Habig *et al.,* (1974). The reaction mixture consisted of 0.067 mM GSH, 0.067 nm 1-chloro-2,4-dinitrobenzene (CDNB), 0.1 M phosphate buffer (pH 6.0) and 0.1 ml of post-mitochondrial supernatant in a total volume of 3 ml. Absorbance was read at 340 nm for 10 min every 30 sec by an optical plate reader and the enzyme activity was calculated as mMol CDNB conjugate formed min^{-1} mg^{-1} protein using a molar extinction coefficient of $9.6\times10^3 M^{-1}cm^{-1}$.

Estimations of GPx activity in liver

Glutathione peroxidase activity was modified from the method of Flohe and Gunzler, (1984). For the enzyme reaction, 0.2 mL of the post-mitochondrial supernatant was placed into a tube and mixed with 0.4 mL reduced glutathione and the mixture was put into an ice bath for 30 min. Then the mixture was centrifuged for 10 min at 3000 rpm, 0.48 ml of the supernatant was placed into a cuvette, and 2.2 ml of 0.32 MNa_2HPO_4 and 0.32 ml of 1.0 mMol/L DTNB were added for color development. The absorbance at wavelength 412 nm was measured on spectrophotometer (LKB-Pharmacia, Mark II, Ireland) after 5 min. The enzyme activity was calculated as nMol/mg protein.

Statistical analysis

All data were presented as the mean ± Standard Deviation (SD). The data were evaluated by a one-way ANOVA using Graph Pad Prism program and the differences between means were assessed using Student Newman-Keuls. The differences were considered statistically significant at $P<0.05$.

RESULTS

Mean final body weights were significantly ($P<0.001$) decreased in diabetic rats while liver, kidney and spleen weights showed significant ($P<0.001$) increase when compared to control group. Body weights of the diabetic rats supplemented with SM (60 mg/kg/day) were found significant ($p<0.05$) increase when compared to untreated diabetic rats. The combined treatment of RT and SM produced more significant ($P<0.01$) effect of body weight compared to STZ. Liver and spleen weights were significantly decreased in RT ($P<0.01$) and SM ($P<0.001$) supplemented diabetic rats and their values become normal when the combined (RT+SM) therapy used in diabetic rats. Mean kidney weights only found significant ($P<0.01$) decrease in RT+SM+STZ group compared to STZ (Table 1).

Serum fasting glucose levels significantly ($P<0.001$) increased while insulin levels were decreased in STZ-induced diabetic rats. RT and SM individually showed hypoglycemic effect as glucose levels decreased ($P<0.05$), however, this effect found more significant ($P<0.001$) when combination of RT and SM was used on glucose levels. Similar effect of combined therapy was seen on insulin levels (Table 2).

Serum enzyme levels including AST, ALT, ALP and ACP were significantly ($P<0.001$) elevated in diabetic rats and the values markedly decreased in RT and SM treated diabetic rats but the levels of these enzymes more significantly ($P<0.001$) inhibited when RT and SM were supplemented simultaneously to diabetic animals. Total protein and albumin levels were significantly ($P<0.001$) decreased in diabetic rats while CRP levels increased as compared to control group. Six weeks RT, SM and RT+SM treatments to diabetic rats, TP and albumin levels significantly increased and CRP levels decreased when compared to untreated diabetic rats. However, the treatment effect found higher in combined therapy group (RT+SM+STZ) when compared to

RT+STZ and SM+STZ respectively. Serum lipid profile including TC and TG levels were significantly increased in 9 weeks of diabetic rats. Six weeks treatments of RT and SM produced significant P<0.05 and p<0.01 inhibition in TC and TG levels respectively. The combined therapy, showed more significant (P<0.001) effect against hyperlipidemia-induced by diabetic in rats (Table2). Pro-inflammatory biomarkers including TNF-α, IL-6 and IL-1β levels were significantly increased in diabetic rats compared to control animals. Treatments with RT or/and SM to diabetic rats markedly inhibited the elevated levels of TNF-α, IL-6 and IL-1β compared to untreated diabetic animals. However, the potential anti-inflammatory effect of both the compounds was found higher when they combined supplemented to the diabetic rats (Fig. 1).

In diabetic rats, significantly (P<0.001) increased TBARS and decreased GSH levels compared to control animals. RT or/and SM supplementations to diabetic rats were markedly decreased the changes as compared to untreated diabetic animals. The combined therapy of RT and SM showed more potential effect (P<0.001) on LPO-induced by the diabetes in rats. The enzymatic activities including SOD, CAT, GST and GPx were significantly (P<0.001) reduced in diabetic rats as compared to control animals. Treatments with RT and SM markedly enhanced these enzymatic activities in diabetic rats. However, the diabetic-induced inhibition in enzymatic activities were more significantly (P<0.001) enhanced by the combined treatment with RT and SM compared with individual treatment (Fig. 2).

Table. 1: Effect of rutin (RT) or/and silymarin (SM) on diabetic-induced changes in organ weights corresponding with body weight.

Treatments	Body weight increased (g)	Organ weight/100 g body weight		
		Liver	Kidney	Spleen
Control	85.17±21.13	2.78±0.23	0.27±0.03	0.27±0.02
STZ	0.33±11.23^{***a}	3.94±0.36^{***a}	0.39±0.03^{***a}	0.48±0.06^{***a}
RT(100)+STZ	12.01±8.32	3.35±0.26^{**b}	0.37±0.02	0.39±0.05^{**b}
SM(60)+STZ	19.32±9.37^{*b}	3.10±0.39^{***b}	0.35±0.02	0.34±0.06^{***b}
RT(50)+SM(30)+STZ	22.54±10.32^{**b}	2.76±0.31^{***b}	0.32±0.03^{**b}	0.30±0.03^{***b}

One-way ANOVA and Student-Newman-Keuls multiple comparisons test was applied
'a' Control vs STZ and 'b' RT(100)+STZ, SM(60)+STZ and RT(50)+SM(30)+STZ vs STZ
*P<0.05, **P<0.01 and ***P<0.001 (n=6, Mean±S.D.)

Table. 2: Effect of rutin (RT) or/and silymarin (SM) on diabetic-induced changes in serum biochemical markers.

Parameters	Control	STZ	RT(100)+STZ	SM(60)+STZ	RT(50)+SM(30) + STZ
Glucose (mg/dl)	119.50±3.58	392.28±41.63^{***a}	348.80±39.38^{*b}	339.16±25.62^{*b}	306.76±39.38^{***b}
Insulin (µU/ml)	19.37±1.62	12.27±3.04^{***a}	14.83±1.98	16.24±2.65^{*b}	18.82±2.38^{***b}
AST (U/L)	40.00±3.59	104.83±11.88^{***a}	88.76±10.40	79.85±7.15^{*b}	67.48±24.87^{***b}
ALT (U/L)	23.36±2.81	68.66±14.87^{***a}	53.65±14.41^{*b}	47.06±8.75^{**b}	42.74±12.74^{***b}
ALP (U/L)	238.83±18.89	365.89±31.77^{***a}	336.31±27.37^{*b}	302.20±15.81^{***b}	278.65±24.56^{***b}
ACP (U/L)	32.08±3.38	58.35±16.48^{***a}	49.71±5.77	38.50±9.87^{**b}	34.51±14.56^{***b}
T. Protein (mg/dl)	9.84±1.20	6.74±0.45^{***a}	7.29±0.81^{*b}	8.56±1.01^{**b}	8.84±0.57^{***b}
CRP (mg/dl)	2.80±0.29	9.90±1.57^{***a}	8.27±1.57^{*b}	7.65±0.98^{**b}	6.46±1.15^{***b}
Albumin (mg/dl)	40.23±6.25	25.12±5.95^{***a}	32.87±2.80^{*b}	35.57±3.87^{**b}	37.46±3.68^{***b}
Triglyceride(mg/dl)	62.92±8.00	106.89±13.77^{***a}	94.33±9.18^{*b}	85.96±8.86^{**b}	75.45±4.51^{***b}
T. cholesterol (mg/dl)	52.07±9.33	108.92±16.27^{***a}	88.32±9.87^{*b}	82.83±14.08^{**b}	74.14±14.65^{***b}
Creatinine (µmol/L)	36.28±5.75	108.74±14.13^{***a}	87.13±15.01^{**b}	79.47±12.04^{***b}	68.91±7.95^{***b}
BUN (mmol/L)	10.08±1.10	29.71±5.14^{***a}	24.18±4.78^{*b}	22.65±5.86^{*b}	19.55±3.98^{**b}

One-way ANOVA and Student-Newman-Keuls multiple comparisons test was applied
'a' Control vs STZ and 'b' RT(100)+STZ, SM(60)+STZ and RT(50)+SM(30)+STZ vs STZ
*P<0.05, **P<0.01 and ***P<0.001 (n=6, Mean±S.D.

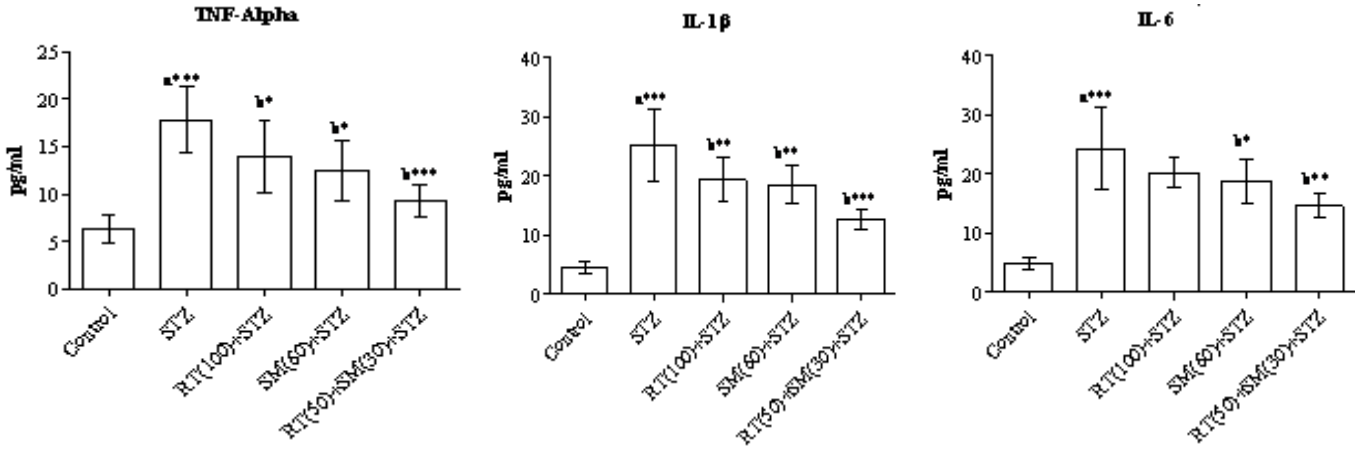

Fig. 1: Effect of rutin (RT) or/and silymarin (SM) on diabetic-induced changes in serum pro-inflammatory biomarkers including TNF-α, IL-1β and IL-6 levels One-way ANOVA and Student-Newman-Keuls multiple comparisons test was applied. 'a' Control vs STZ and 'b' RT(100)+STZ, SM(60)+STZ and RT(50)+SM(30)+STZ vs STZ. *P<0.05, **P<0.01 and ***P<0.001 (n=6, Mean±S.D.)

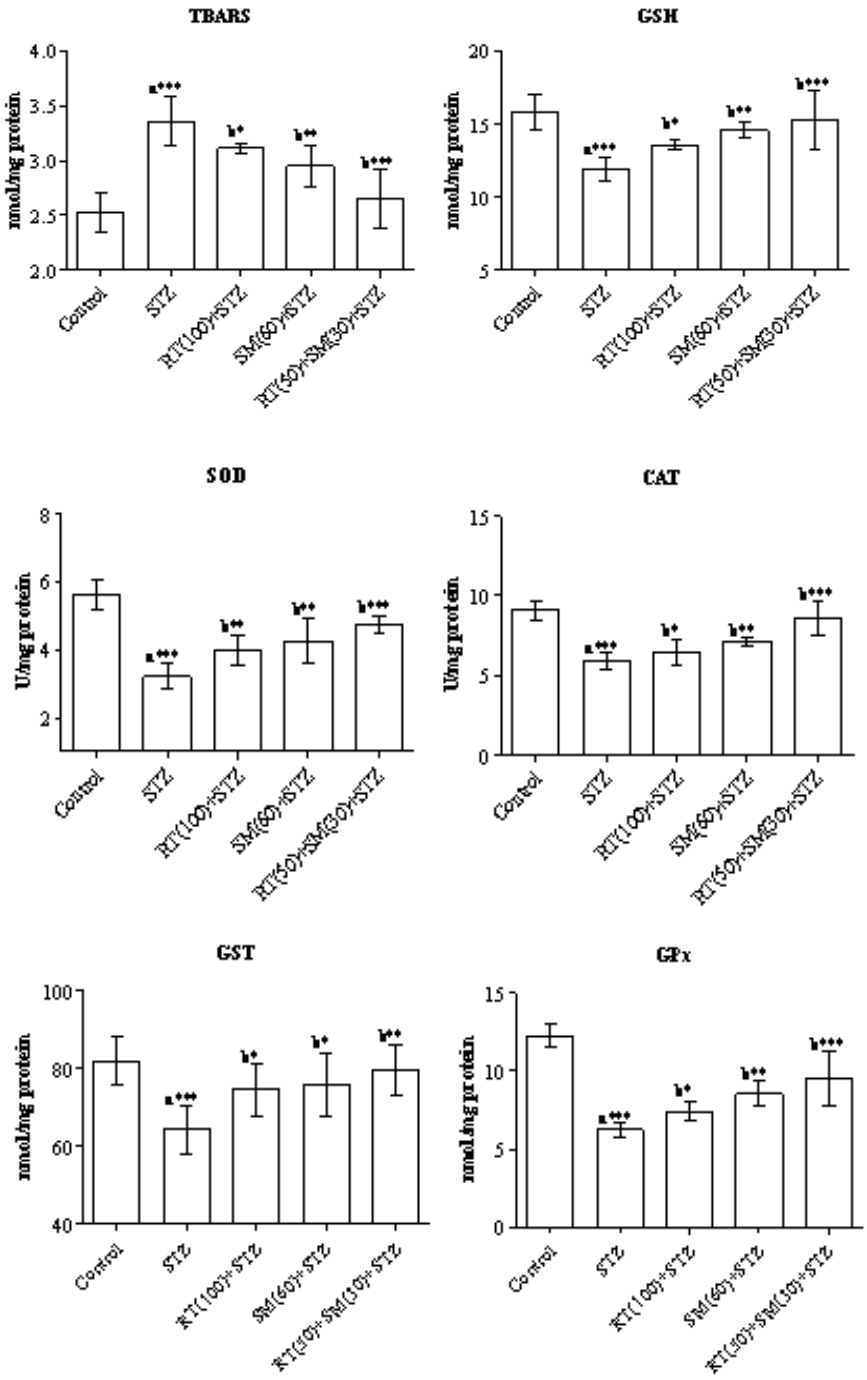

Fig. 2: Effect of rutin (RT) or/and silymarin (SM) on diabetic-induced changes in TBARS and GSH levels and in SOD, CAT, GST and GPx activities in hepatic cells. One-way ANOVA and Student-Newman-Keuls multiple comparisons test was applied. 'a' Control vs STZ and 'b' RT(100)+STZ, SM(60)+STZ and RT(50)+SM(30)+STZ vs STZ. *P<0.05, **P<0.01 and ***P<0.001 (n=6, Mean±S.D.).

DISCUSSION

Present study was designed to elucidate the relationship between diabetic-induced oxidative stresses by following the parameters related to hepatic function in the rats. Our results revealed that STZ-injection causes significant alterations in the body composition. The serum levels of different biomarkers were markedly altered by the STZ-induced diabetes and the treatments with RT and/or SM to diabetic rats for 5 weeks corrected the levels to normal values. Diabetes induced significant oxidative impairments as evidenced by LPO biomarkers such as MDA levels increased and GSH decreased in the hepatic cells. Antioxidant enzyme activities including SOD, CAT, GPx and GST were significantly inhibited in hepatic cells of diabetic rats and in

contrast, pro-inflammatory biomarker like IL-6, IL-1β and TNF-α level were markedly elevated. The above diabetic-induced changes were significantly ameliorated by the RT or/and SM treatment. However, the combined therapy of RT and SM produced more significant effect against the diabetic-induced changes.

Several studies have shown an association between hyperglycemia and decreased body weight of diabetic animals (Zafar and Naqvi, 2010; Okon *et al.,* 2012). It also described that the organ weights increases in diabetic animals (Alsiaf, 2009; Lee *et al.,* 2008; Ramadan *et al.,* 2009). Similar results have seen in the present study, boy weights of diabetic animals were significantly decreased and their organ weights including liver, kidney and spleen were increased in STZ group. These changes were normalized by the combined treatment of RT and SM contributing to their synergistic anti-inflammatory and anti-oxidative properties. Increase in serum levels of AST and ALT shows hepatic injuries similar to viral hepatitis, infarction, and muscular damages (Drotman & Lawhorn, 1978). In addition, ALP is membrane bound and its alteration is likely to affect the membrane permeability and produce derangement in the transport of metabolites (Mehana *et al.,* 2012). In present study, these enzymes were significantly increased in diabetic rats. Combined treatment with RT and SM to diabetic rats more significantly inhibited the increased levels of enzymes than individual treatment. CRP, an acute phase reactant, produced mainly by liver and selectively binds to LDL, is found within atheromatous plaques and enhances complement activation (Pradhan *et al.,* 2001). In a similar study, Goyal and coworkers found that STZ-administered group possessed high level of CRP, an important cardiac biomarker (Goyal *et al.,* 2011). We also found a similar rise in CRP level by the STZ-injection and that was later corrected by the RT and SM treatment. Angiotensin II (Ang II) has significant proinflammatory actions in the vascular wall, inducing the production of ROS, inflammatory cytokines, and adhesion molecules, elevating the CRP levels. Enhanced production of angiotensinogen, in turn, supplies more substrate to the activated vascular ras angiotensin system (RAS), wherein the locally produced Ang II synergizes with oxidized lipid to perpetuate atherosclerotic vascular inflammation and increases CRP levels (Goyal *et al.,* 2008). Several natural products including phenolic compounds are known to act as ACE-inhibitor inhibit Ang II induced proinflammation and thereby decreases the CRP levels (da Silva Pinto *et al.,* 2008; Apostolidis *et al.,* 2006). The levels of serum lipids are usually elevated in diabetes mellitus (Ramesh *et al.,* 2006), similar increase was found in present study. RT or/and SM reinstated the elevated lipid levels again the effect found higher in combined therapy group. The individual lipid lowering effect of these phenolic compounds have been reported in earlier studies (Khan *et al.,* 2012; Alsaif, 2009; Ramakrishnan *et al.,* 2008).

Hyperglycemia results in the generation of free radicals which can exhaust antioxidant defenses thus leading to the disruption of cellular functions, oxidative damage to membranes and enhanced susceptibility to LPO (Giugliano *et al.,* 1996). Lipid peroxides and hydroperoxidesare the secondary products of oxidative stress and are unleashed as a result of the toxic effect of ROS produced during LPO in diabetes (Karthikesan *et al.,* 2010). In the present study, STZ treatment significantly increased LPO product (MDA) and decreased enzymatic and non-enzymatic antioxidant levels in the liver of rats. Elevated LPO in STZ-induced diabetes is the reduction in the levels of reduced glutathione, a potent endogenous antioxidant. Insulin secretion is also closely associated with lipoxygenase-derived peroxides (Maritim *et al.,* 2003). Low levels of lipoxygenase peroxides stimulate the secretion of insulin, but when the concentration of endogenous peroxides increases, it may initiate uncontrolled LPO leading to cellular infiltration and islet cell damage. The observed increase in the level of TBARS in diabetic rats is generally thought to be a consequence of increased production and liberation into the circulation of tissue lipid peroxides due to pathological changes. The increased LPO during diabetes, as found in the present study may be due to the inefficient antioxidant system prevalent in diabetes (Parveen *et al.,* 2010). Administration of RT or/and SM to diabetic rats significantly decreased the levels of TBARS. Both the compounds act as antioxidant by scavenging free radicals which result in decreased LPO in diabetic rats however the combined therapy showed more potentials than individual.

The present study indicated an association between a variety of inflammation markers and the development of diabetic neuropathy. The promoter polymorphism in the TNF gene has been implicated in the regulation of TNF-α production and has been associated with a wide spectrum of inflammatory and infectious diseases. Furthermore, increased levels of inflammatory mediators such as proinflammatory cytokines (IL-6 and IL-1β) have been reported in diabetic states to be a consequence of hyperglycemia (Brownlee, 2005) and these mediators have been considered to be the link between inflammation and insulin resistance (Navarro-Gonzalez & Mora-Fernandez, 2008). In present study, serum proinflammatory markers including TNF-α, IL-6 and IL-1β are significantly increased in STZ-induced diabetic rats. Treatment with RT or/and SM to the diabetic rats significantly reduced these markers where as the combined therapy produced higher effect. This may be because of RT and SM showed antioxidant and anti-inflammatory properties in earlier studies (Abu-El-Fattah *et al.,* 2010; Alsaif, 2009; Miroliaee *et al.,* 2011; Hussain *et al.,* 2009). Antioxidants constitute the foremost defense system that limit the toxicity associated with free radicals. Oxidative stress in diabetes is coupled with decrease in the antioxidant status, which can increase the deleterious effects of free radicals (Atli *et al.,* 2004). Antioxidant enzymes form the first line of defense against ROS in the organism includes the enzymes SOD, CAT, GPx and GST, which play an important role in scavenging the toxic intermediate of incomplete oxidation. SOD and CAT are the two major scavenging enzymes that remove radicals in vivo. A decrease in the activities of these antioxidant enzymes can lead to an excess availability of O_2 and H_2O_2, which in turn generate OH, resulting in initiation and propagation of LPO. SOD protects tissues against oxygen free radicals

byscavenging O_2, which damages the membrane and biological structures (Arivazhagam *et al.,* 2000). SOD can catalyze dismutation of O_2 into H_2O_2, which is then deactivated to H2O by CAT or GPx (Murugan and Pari, 2006). Thus, SOD can act as a primary defense against O_2 and prevents further generation of free radicals. The activity of SOD was found to be lower in diabetic subjects. The observed decrease in SOD activity could result from inactivation by H_2O_2 or by glycation of the enzyme, which have been reported to occur in diabetes (Sozmen *et al.,* 2001). CAT is a hemeprotein, which is present virtually in all mammalian cells and is responsible for the reduction of H_2O_2 and protects tissues from highly reactive OH radicals (Sozmen *et al.,* 2001). The decrease in CAT activity could also result from inactivation by glycation of the enzyme. Any compound, natural or synthetic, with antioxidant properties might contribute towards the partial or total alleviation of oxidative damage. Therefore removing O_2 and OH is probably one of the most effective defenses against diseases (Sankaranarayanan and Pari, 2011). Treatment with RT or/and SM increased the activities of SOD and CAT in diabetic rats when compared with diabetic control rats. In fact, the reactivation in SOD activity promoted by RT and/or SM may accelerate the disputation of O_2 to H_2O_2, which is quickly removed by CAT protecting the hepatic and renal tissues of diabetic rats against highly reactive and toxic OH and consequently preventing the LPO. The increased activities of antioxidant enzymes may act as an added compensation mechanism to maintain the cell integrity and protection against free radical damage. This showed that free radical scavenging ability of RT or/and MS could exert a beneficial action against pathogenic alterations caused O_2 and OH. From the above findings, we conclude that RT and SM have the ability to ameliorate oxidative stress in plasma and tissues of STZ-induced diabetic rats as evidenced by improved glycemic and antioxidant status along with decreased lipid peroxidation. However the present results revealed that the combined therapy of these phenolic compounds is more effective than individual usage particularly in diabetic-induced metabolic changes. Thus, combined therapy of RT and SM should be considered as a treatment strategy for diabetic condition in humans.

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