

Full Length Research Paper

***In vitro* antioxidant effects of *Trigonella foenum graecum* (L.) Fenugreek seed extract on sheep red blood cells**

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Accepted 11 June, 2012

An extract from the seeds of *Trigonella foenum graecum* (Fenugreek) was evaluated for its protective effect against hydrogen peroxide (H₂O₂) induced oxidation in normal sheep erythrocytes (red blood cells (RBCs)). RBCs, treated with increasing concentrations of 30% H₂O₂ along with fenugreek seed extract (FSE) were analyzed for hemolysis and lipid peroxidation. Sheep RBCs treated with increasing concentrations of glucose, to study effect of high glucose level or hyperglycemia on normal SRBC, were found to be more susceptible to lipid peroxidation than those from normal subjects. However, on treatment with FSE, the oxidative modifications in both the groups (RBCs treated with glucose and not treated with glucose) were found to reduce significantly. The inhibition of lipid peroxidation was concentration-dependent up to 100 µl of extract, which contained 0.75 mM gallic acid equivalent (GAE) of phenolic compounds. The total phenolic content in the extract was determined spectrophotometrically according to the Folin-Ciocalteu procedure and was expressed as mg or mM GAE. The results indicate that the extract of fenugreek seeds contains antioxidants and protects cellular structures from oxidative damage. These findings demonstrate the potent antioxidant properties of the fenugreek seeds.

Key words: Fenugreek seed extract (FSE), type II diabetes, osmofragility test, lipid peroxidation.

INTRODUCTION

Many traditional plants were being investigated for their potential as a source of new hypoglycemic compounds for the treatment of diabetes or any condition that leads to hyperglycemia. *Trigonella foenum-graecum* (Fenugreek) plant has been traditionally used as a condiment in the Indian sub-continent and in Mediterranean

countries. Extracts of seeds and leaves of fenugreek are shown to possess hypoglycemic activity and have been proved to be nontoxic (Abdel-Barry et al., 1997; Zia et al., 2001; Hannan et al., 2007).

Several investigators have proved the hypoglycemic and hypolipidemic effects of fenugreek seeds *in vivo* and *in vitro*. Treatment with fenugreek seed extract (FSE) decreased blood glucose levels and serum lipids in type I diabetic (Sharma et al., 1990) and in type II diabetic patients (Sharma et al., 1996; Madar et al., 1988). FSE specifically activates the insulin receptor (IR) and its downstream signalling molecules in adipocytes and liver cells in alloxan induced diabetic mice. FSE was also shown to improve glucose homeostasis in alloxan diabetic rat tissues by reversing the altered glycolytic, gluconeogenic, and lipogenic enzymes (Raju et al., 2001).

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Abbreviations: GAE, Gallic acid equivalent; TBARS, thiobarbituric acid reducing substances; LPO, lipid peroxidation; FSE, fenugreek seed extract; SOD, superoxide dismutase; PBS, phosphate buffered saline; Gpx, glutathione peroxidase; GSH, glutathione reductase.

Several evidences suggest that oxidative stress plays a major role in the pathogenesis of both types of diabetes mellitus. Damage to red blood cell (RBC) membrane lipids and proteins is caused by excessively high levels of free radicals, which will finally result in hemolysis (Lubin and Chiu, 1982). Formation of these reactive oxygen-free radicals (ROS) is due to various mechanisms. One of the main sources of free radicals is the oxidation of glucose. Hyperglycemia, seen in conditions like diabetes mellitus, is found to promote lipid peroxidation of low density lipoproteins (LDL) resulting in the generation of free radicals, responsible for change in a number of RBC properties. Another important source of free radicals in diabetes is the interaction of glucose with proteins leading to the formation of an intermediate product followed by advanced glycation end products (AGEs) (Hori et al., 1996; Mullarkey et al., 1990). The superoxide anion radicals in the circulation undergo dismutation to form hydrogen peroxide (H₂O₂). Hydrogen peroxide, if not degraded by catalase or glutathione peroxidase, can lead to the production of extremely reactive hydroxyl radicals in the presence of transition metals (Jiang et al., 1990; Wolff et al., 1987). In addition, oxidative damage can cause immune recognition of RBCs (Low et al., 1985).

FSE contains factor(s) that might act independently of insulin to enhance transporter-mediated glucose uptake (Vijayakumar et al., 2005). Handa et al. (2005) showed hypolipidemic effect in high fat diet fed obese mice. Polyphenol extract of fenugreek seeds showed protective effect against oxidative damage in RBCs (Kaviarasan et al., 2004).

This study was designed to determine the protective effect of FSE on RBCs treated with high glucose concentrations that induces hyperglycemic condition *in vitro*. In this study, the extent of damage to RBCs exposed to increasing concentrations of glucose was evaluated by membrane fragility test. In addition, oxidative damage due to different glucose concentrations was evaluated by comparing lipid peroxidation levels and activities of antioxidant enzymes, namely, catalase, superoxide dismutase, and glutathione peroxidase, respectively.

MATERIALS AND METHODS

In the present study, sheep red blood cells (SRBCs) prepared from heparinised blood collected from a slaughter house in Puducherry was used. Fenugreek seeds were purchased from Nilgiris Super Market in Puducherry. All the chemicals used in the assays were of analytical grade and were purchased from Hi media, Sigma chemicals and Qualigens, Mumbai, India.

Preparation of FSE

Fenugreek seeds (100 g) were cleansed, dried at 40°C and finely powdered. The powder was then mixed with absolute ethanol and was kept for 5 days. After incubation with absolute ethanol, the solvent was filtered and then evaporated under the hood. The

residue was dissolved in water and the aqueous layer was washed with petroleum ether several times until a clear upper layer of petroleum ether was obtained. The lower layer was then treated with ethyl acetate containing glacial acetic acid (10 ml/L). Extraction was then carried out for 36 h at room temperature and the combined ethyl acetate layer was concentrated (Xia et al., 1998). The dried FSE obtained after evaporation of the solvent was dissolved in sterile MilliQ water and was stored at -20°C until usage. This yielded about 2 to 3 g per 100 g of seed powder. An aqueous extract was prepared and used for *in vitro* studies. The polyphenolic content of the extract was assessed by the method of Singleton and Rossi (1965) and expressed as mg gallic acid equivalents (GAE) per gram of dry extract, using a standard curve generated with gallic acid.

Osmotic fragility test

Preparation of sample

One volume of the collected sheep blood sample was centrifuged. RBCs that settled in the pellet were then washed and suspended in sterile 1X phosphate buffer saline solution (pH 7.4). The suspended RBCs were then incubated with increasing concentrations of 30% H₂O₂, namely, 10, 20, and 50 mM for 2 h. The other volume of blood was first incubated with 100 µl of FSE for 1 h. The blood was centrifuged. The RBCs that settled in the pellet were then washed and suspended in phosphate buffered saline (PBS) (pH7.4). The suspended RBCs were incubated with increasing concentrations of H₂O₂, namely, 10, 20, and 50 mM for 2 h.

The percentage hemolysis was evaluated by performing osmotic fragility test as described by Dacie et al., (1964).

Treatment with glucose and fenugreek

SRBCs prepared from fresh blood samples were treated with three different concentrations (namely, 20, 30, and 45 mM) of glucose for 6 h to induce hyperglycemic condition *in vitro* and a second set of blood samples without treatment served as control. Simultaneously, a third set of SRBCs were treated similarly with three different concentrations of glucose along with 100 µl of FSE (with a concentration of 0.75 mM GAE of phenolic compounds).

Determination of activity of antioxidant enzymes

Estimation of hemolysate protein

The concentration of protein in the hemolysate was estimated by the standard method of Lowry et al. (1951).

Preparation of sample

One volume of collected sheep blood was incubated with increasing concentrations of glucose, namely, 20, 30, and 45 mM for 6 h. The other volume of blood was incubated with increasing glucose concentrations along with 100 µl of FSE for 6 h. Each of the incubated samples was hemolysed separately. The hemolysed blood was centrifuged at 20,000 g in a high speed centrifuge for 40 min. The supernatant or hemolysate was used for the assay. The sedimented membrane might be washed with PBS (pH 7.4) and used for estimation of membrane bound enzymes. A third volume of blood was preincubated with 100 µl of FSE for 1 h. The RBCs were then washed and incubated with 50 mM H₂O₂ for 2 h. A fourth volume of blood sample was incubated only with 50 mM H₂O₂ for 2 h. Both the samples were hemolysed separately and this was used

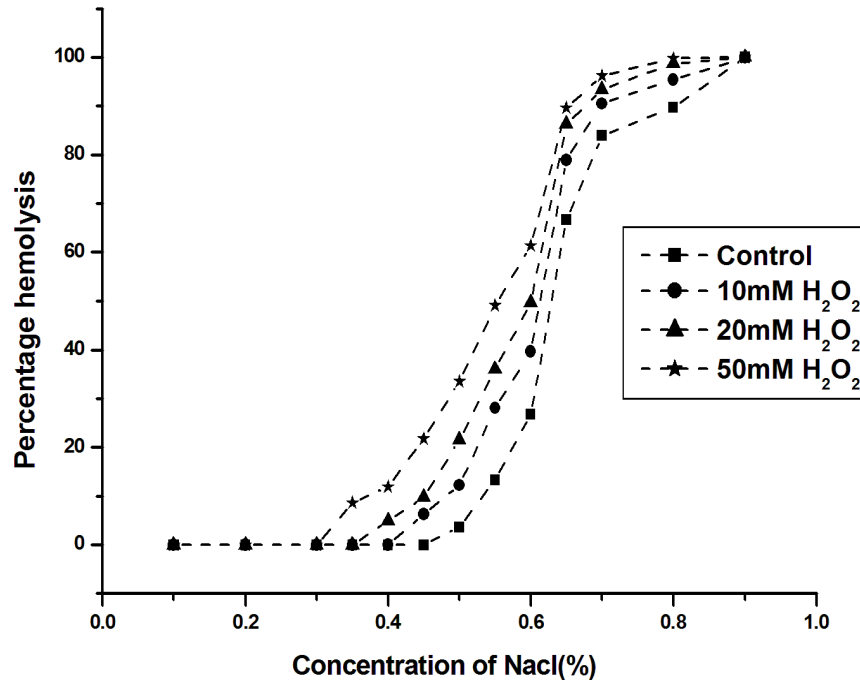


Figure 1. Rate of increasing H₂O₂ concentrations induced RBC hemolysis. Values shown are mean \pm SD (n = 6).

as the positive control.

The supernatant from the lysed blood was used immediately to estimate the levels of thiobarbituric acids (TBARS) and the activities of antioxidant enzymes, namely, catalase, superoxide dismutase (SOD), and glutathione peroxidase, respectively. Catalase was estimated by the method of Sinha (1972). Superoxide dismutase was assayed by the method of Marklund and Marklund (1974). Glutathione peroxidase was estimated by the method of Rotruck et al. (1973) with some modifications.

Statistical methods

Results are expressed as mean \pm standard deviation (SD). Statistical analysis was done by one way analysis of variance (ANOVA), followed by multiple comparison by Tukey's honestly significant difference (HSD) test. The values were considered statistically significant when $p < 0.05$.

RESULTS

Positive effect of FSE on the RBC membrane integrity

Results obtained in osmofragility test showed that there was no significant increase in the rate of hemolysis in 10 mM H₂O₂ treated blood sample as compared to the control sample suggesting that there was no significant membrane damage at this concentration. With 20 and 50mM H₂O₂ concentrations, the rate of hemolysis was found to increase as depicted by the osmofragility curves that deviated significantly from the control curve.

Maximum hemolysis was observed in blood incubated with a concentration of 50 mM H₂O₂ (Figure 1). Hemolysis was found to decrease in blood incubated with FSE along with H₂O₂ suggesting that fenugreek has some protective effect in maintaining the RBC membrane integrity (Figures 2, 3, and 4).

Effect of high glucose concentrations and fenugreek treatment on the levels of thiobarbituric acid reactive substances

Lipid peroxidation (LPO) level in RBCs treated with glucose increased as could be seen from significantly elevated levels of TBARS in treated samples when compared with that of the control group (Figure 5). Treatment with FSE decreased the levels of TBARS significantly in both glucose treated and untreated RBCs. Lipid peroxidation was found to be maximum in RBCs treated with 45 mM glucose concentration and there was a significant decrease ($p < 0.05$) in lipid peroxidation in RBCs treated with FSE along with a particular concentration of glucose.

Effect of high glucose concentrations and fenugreek treatment on catalase activity

Catalase activity was found to increase significantly on

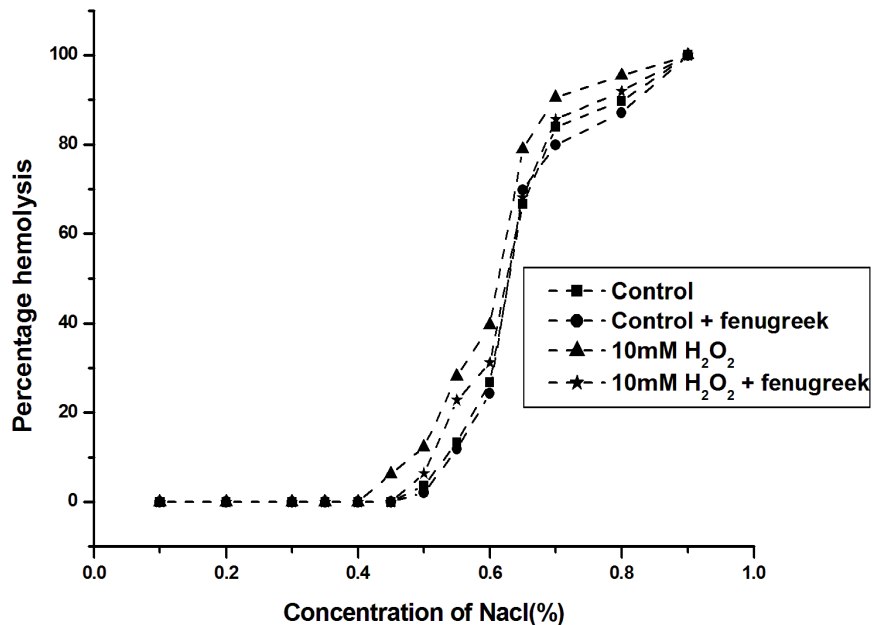


Figure 2. Rate of RBC hemolysis in control and 10 mM H₂O₂ treated blood with and without preincubation with 100 μl fenugreek seed extract. Values shown are mean ± SD (n = 6).

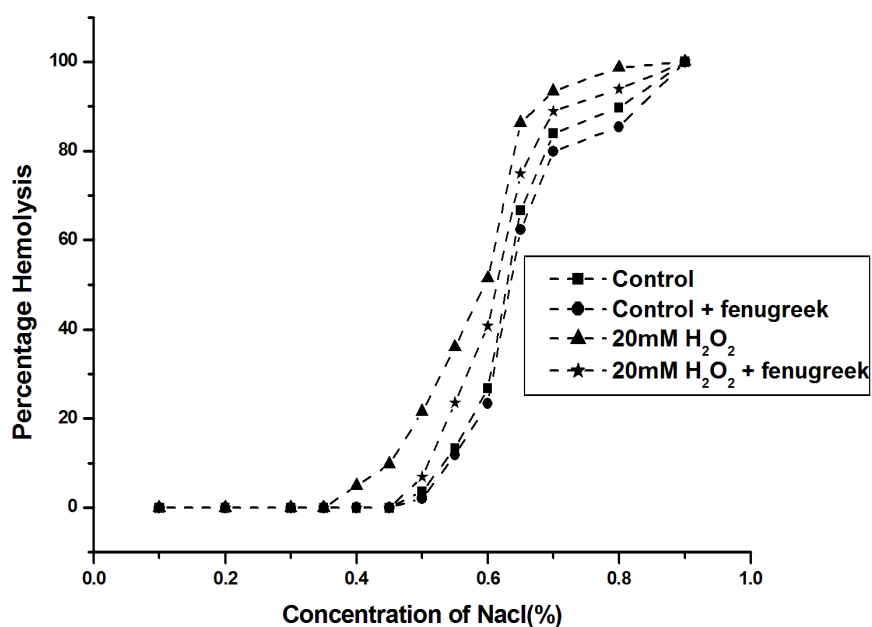


Figure 3. Rate of RBC hemolysis in control and 20mM H₂O₂ treated blood with and without preincubation with 100μl fenugreek seed extract. Values shown are mean ± S.D (n = 6).

exposure to 30 and 45 mM glucose concentrations ($p < 0.05$), but there was no significant change in activity at 20 mM glucose concentration. Catalase activity in RBCs exposed to 50 mM H₂O₂ served as a positive control in

this study. Treatment with FSE along with high glucose brought about significant increase in catalase activity ($p < 0.05$). Similar increase in catalase activity was observed in RBCs treated with 50 mM H₂O₂ and FSE (Figure 6).

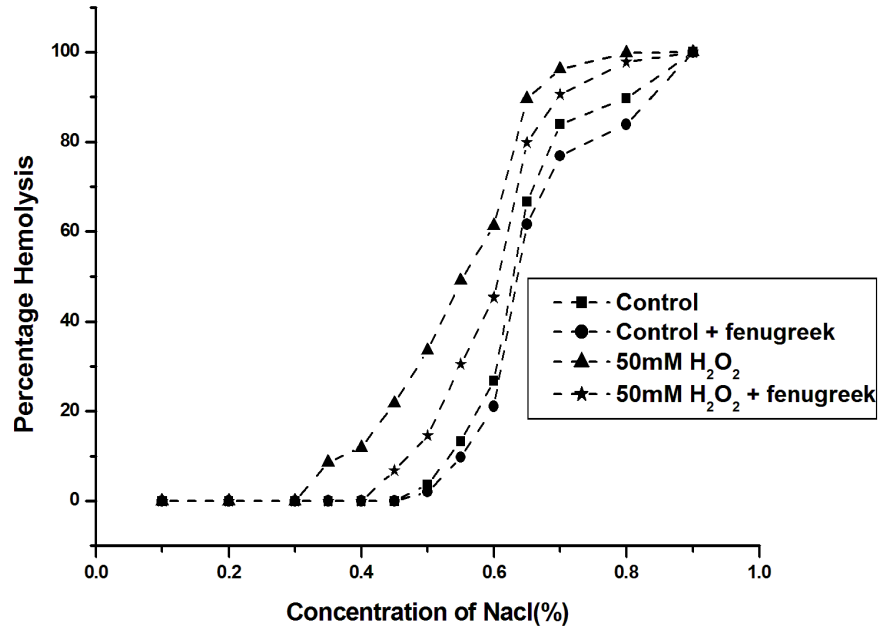


Figure 4. Rate of RBC hemolysis in control and 50mM H₂O₂ treated blood with and without preincubation with 100µl fenugreek seed extract. Values shown are mean ± S.D (n = 6).

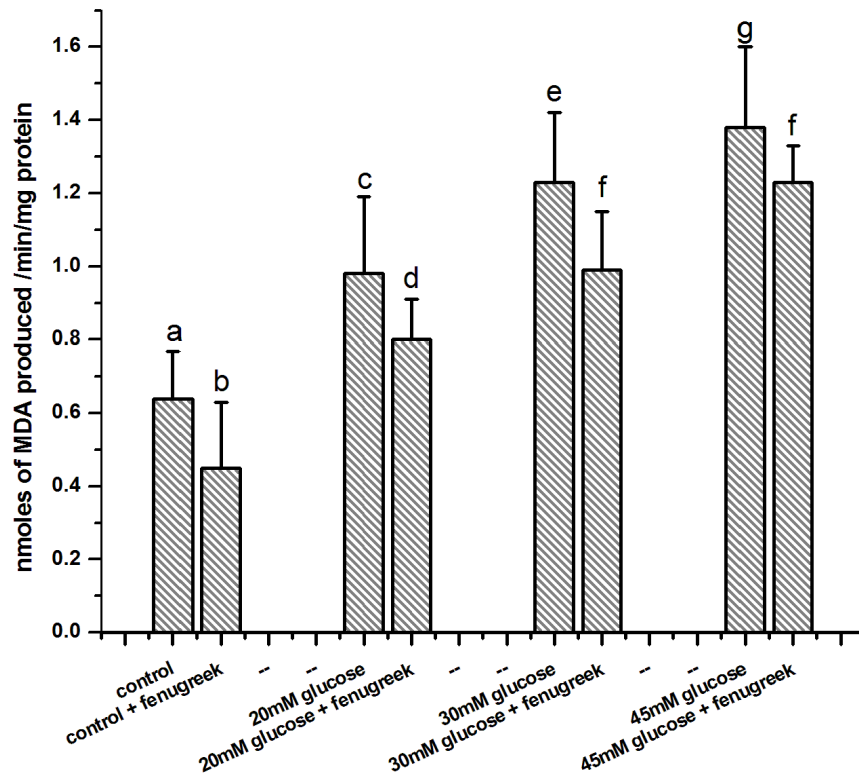


Figure 5. Effect of fenugreek seed extract on the levels of thiobarbituric acid reactive substances (TBARs) in RBCs treated with increasing concentrations of glucose (20, 30 and 45 mM). Values shown are mean ± SD (n = 6). Statistical analysis was done by one way ANOVA and multiple comparison was done by Tukey's HSD test. Values not sharing a common superscript differ significantly. Significance level was p < 0.05.

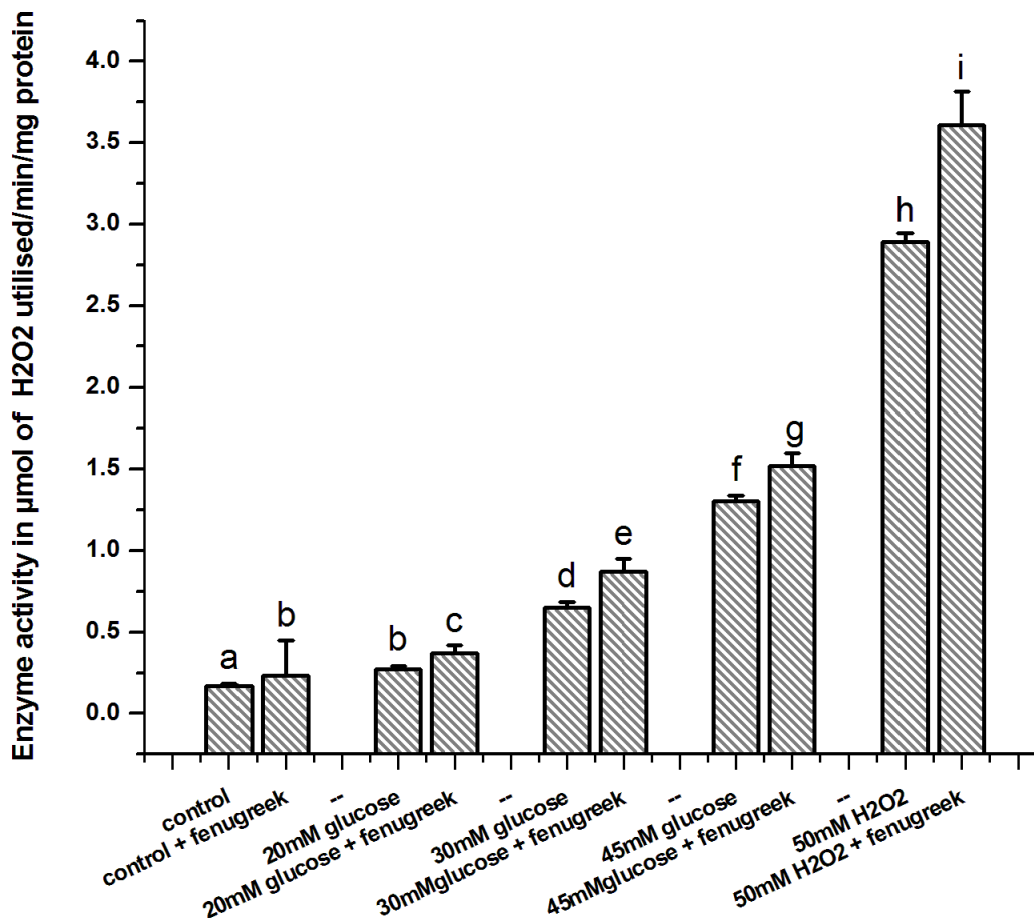


Figure 6. Effect of fenugreek seed extract on catalase activity in RBCs treated with increasing concentrations of glucose (20, 30 and 45 mM). Values shown are mean \pm SD (n = 6). Statistical analysis was done by one way ANOVA and multiple comparison was done by Tukey's HSD test. Values not sharing a common superscript differ significantly. Significance level was $p < 0.05$.

This explains the protective action of fenugreek against oxidative damage on RBCs.

Effect of high glucose concentrations and fenugreek treatment on SOD activity

The activity of super-oxide dismutase was assayed in the hemolysate of RBCs treated with different glucose concentrations (20, 30, and 45 mM) with or without fenugreek treatment. The SOD activity was found to increase significantly on exposure to 30 and 45 mM glucose concentrations ($p < 0.05$), but there was no significant change in the activity at 20 mM glucose concentration. SOD activity in RBCs exposed to 50 mM H₂O₂ served as positive control in this study. Treatment with FSE along with high glucose treatment brought about further increase in SOD activity which was not significant. A significant increase in SOD activity was observed in RBCs treated first with 50 mM H₂O₂ and subsequently with FSE (Figure 7).

Effect of high glucose concentrations and fenugreek treatment on glutathione peroxidase (GPx) enzyme activity

Glutathione peroxidase activity in RBCs treated with different glucose concentrations (20, 30, and 45 mM) with or without fenugreek treatment was determined using the hemolysate. GPx activity was found to increase significantly on exposure to 20, 30, and 45 mM glucose concentrations ($p < 0.05$). GPx activity in RBCs exposed to 50 mM H₂O₂ served as positive control in this study. Treatment with FSE along with high glucose treatment brought about an increase in GPx activity which was not significant. A significant increase in GPx activity was observed in RBCs treated with 45 mM glucose and 50 mM H₂O₂ along with FSE (Figure 8).

DISCUSSION

Hyperglycemia can lead to both an increase in ROS

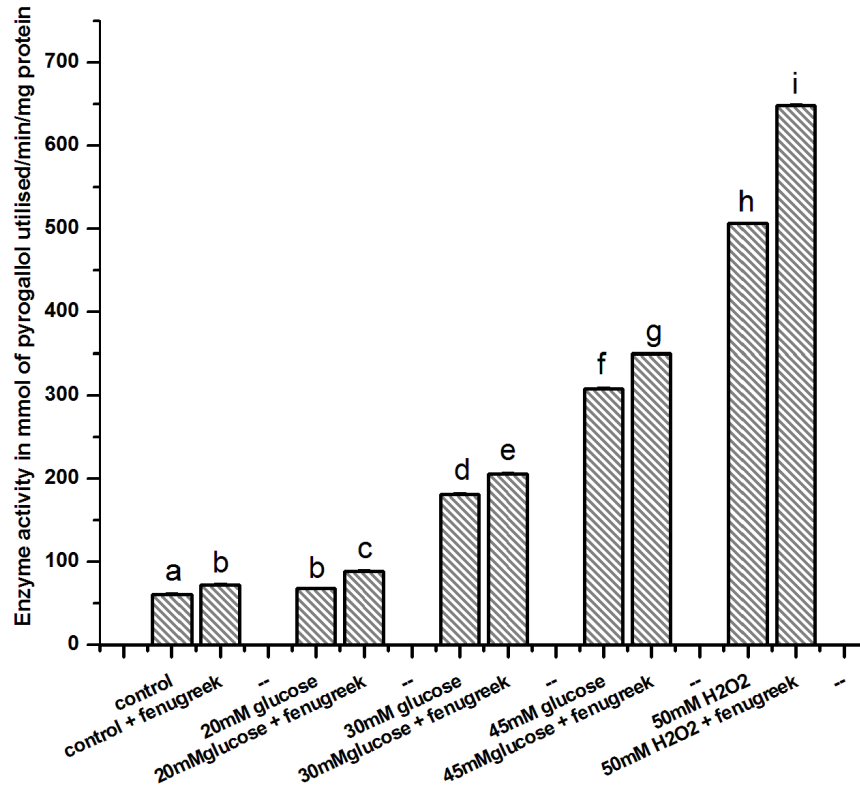


Figure 7. Effect of fenugreek seed extract on superoxide dismutase activity in RBCs treated with increasing concentrations of glucose (20, 30 and 45 mM). Values shown are mean \pm SD ($n = 6$). Statistical analysis was done by one way ANOVA and multiple comparison was done by Tukey's HSD test. Values not sharing a common superscript differ significantly. Significance level was $p < 0.05$.

production and attenuation of free radical scavenging compounds (Baynes, 1991). There are many ways by which hyperglycemia may increase free radical generation, such as glycooxidation, polyol pathway, prostanoid biosynthesis, and protein glycation (Armstrong and Young, 1996). There is also ample evidence that elevation in glucose concentration may depress natural antioxidant defense, such as GSH (Yoshida and Kondo, 1998). The imbalance between the generation of oxygen free radicals and an antioxidant defense system may increase oxidative stress and lead to the damage of molecules, such as DNA, proteins, or lipids.

Using SRBCs as a model, this study demonstrates that FSE can reduce membrane damage due to oxidation in cells exposed to high glucose. High glucose concentrations can induce oxidative stress due to excessive ROS production resulting from the auto oxidation of glucose, glycosylated proteins, or stimulation of cytochrome P450-like activity by the excessive NADPH produced by glucose metabolism (Choudhary et al., 2001). Whereas, FSE reduces membrane damage due to high glucose or increasing concentrations of H₂O₂. FSE, in general, has free radical scavenging property and it can reduce free radicals produced in the presence or

absence of any antioxidant, that is, it can reduce lipid peroxidation levels even in normal red blood cells. Even, reduced osmotic fragility, might be due to increased glucose utilization of the FSE treated cells. Kaviarasan et al. (2004) in their study showed that a decrease in LPO levels in RBCs exposed to increasing concentrations of polyphenol extracts from fenugreek seeds in H₂O₂ induced oxidative damage.

In this study, fenugreek proved to be an effective antioxidant and an anti-inflammatory agent in protecting SRBCs from membrane damage due to high glucose or increasing H₂O₂ concentrations induced oxidative stress. Fenugreek was found to decrease the rate of hemolysis in H₂O₂-treated RBCs, indicating that fenugreek has protective effect in maintaining red blood cell membrane integrity. Fenugreek reduces damage to the fragile RBC membrane due to oxidative stress imposed by H₂O₂. Alterations in LPO levels and antioxidant enzyme activities were observed in blood treated with increasing concentrations of glucose. The levels of TBARs were found to increase on treatment with high glucose concentrations, whereas the activity of antioxidant enzymes was found to increase on long term exposure to oxidative stress. Whereas, in earlier works, the activity of

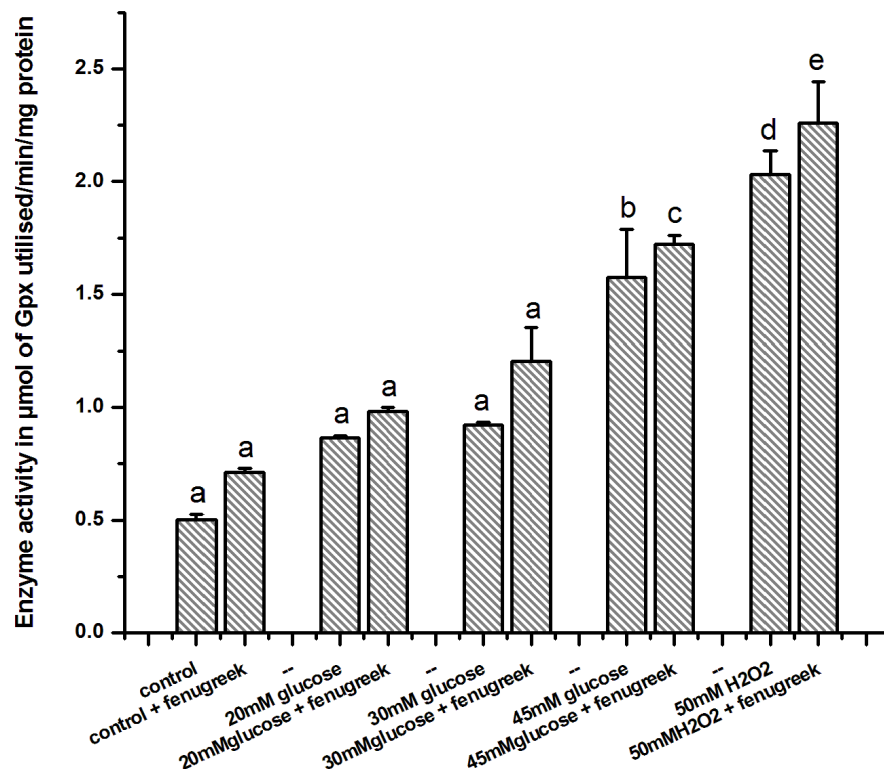


Figure 8. Effect of fenugreek seed extract on glutathione peroxidase activity in RBCs treated with increasing concentrations of glucose (20, 30 and 45 mM). Values shown are mean \pm SD (n = 6). Statistical analysis was done by one way ANOVA and multiple comparison was done by Tukey's HSD test. Values not sharing a common superscript differ significantly. Significance level was $p < 0.05$.

antioxidant enzymes was found to increase initially followed by a drastic decrease later. This decrease in the activity of antioxidant enzymes in some tissues during diabetes may be due to the inactivation or inhibition of the enzymes by the increased production of oxygen free radicals during diabetes (Kakkar et al., 1996; Marklund and Marklund, 1974). In the present work, on prior treatment with FSE, lipid peroxidation levels are found to decrease significantly, whereas the activity of antioxidant enzymes was found to increase further on pretreatment with FSE, indicating that fenugreek reduces oxidative stress to RBCs. The possible source of oxidative stress during hyperglycemic condition includes shifts in redox balance resulting from altered carbohydrate and lipid metabolism, increased generation of reactive oxygen species, and decreased level of antioxidant defenses such as GSH (Baynes 1991). The exact mechanism involved in the activity of fenugreek is not yet known. In the present study, an increase in the level of TBARS and enzymatic antioxidants were observed in blood samples treated with high concentrations of glucose.

The observed results in the present study demonstrate the occurrence of oxidative damage to RBC membranes due to hyperglycemia. The observed increase in lipid peroxidation levels in RBCs are in agreement with similar

findings in rat tissues in earlier studies (Kakkar et al., 1995). Lipid peroxidation may bring about protein damage and inactivation of membrane bound enzymes either through direct attack by free radicals or through chemical modification by its end products, malondialdehyde and 4-hydroxynonenal (Halliwell and Gutteridge, 1999). Glucose was known to induce lipid peroxidation through activation of the lipoxigenase enzymes (Rajeswari et al., 1991). An increased level of TBARS is an index of lipid peroxidation. The present study shows that FSE tends to bring TBARS levels in blood back to near normal, which was increased earlier on exposure to high glucose concentrations. Increased lipid peroxidation under diabetic condition can be due to the increased oxidative stress in the cells as a result of depletion of antioxidant scavenger systems as reported by Anuradha and Selvam (1993). It was also shown that supplementation of seeds in the diet enhances the antioxidant potential in control and in diabetic rats (Anuradha and Ravikumar, 2001).

In the present study, FSE treatment was observed to cause significant decrease in lipid peroxidation. The activities of the antioxidant enzymes, SOD and catalase were observed to increase in glucose treated SRBCs as compared to untreated SRBCs. Higher levels of lipid

peroxides and increased SOD and catalase activity are indicative of an oxidative stress condition. H_2O_2 is toxic by itself and can be a precursor to other toxic species. It can react with $O_2^{\cdot-}$ to form OH^{\cdot} and result in increased lipid peroxidation and hence higher TBARS level (Kono and Fridovich, 1982). This suggests the antioxidant potential of fenugreek seeds which may involve some mechanism related to ROS scavenging activity. Thus, from the results obtained in the present study it can be inferred that fenugreek seeds have potent antioxidant properties. The evidence that fenugreek can prevent oxidative stress needs to be explored further at the clinical level to determine whether supplementation can lower levels of protein glycosylation, circulating glucose levels and oxidative stress and thereby reduce the incidence of vascular disease in the diabetic patient population.

ACKNOWLEDGEMENTS

The authors thank UGC, India for the support through the Major Research Project (F.NO. 36-191/2008 (SR) dated 26 March 2009).

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