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EFFECTS OF THYMOL SUPPLEMENTATION AGAINST TYPE 2 DIABETES IN STREPTOZOTOCIN-INDUCED RAT MODEL

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Abstract

The present investigation was aimed to study the effect of thymol, an active compound of *Nigella sativa*, on streptozotocin (STZ) induced diabetic rats. Thirty male Wistar rats were randomly divided into five groups (i) Normal control, (ii) Diabetic control, (iii) Diabetic treated with insulin (iv) Diabetic treated with thymol and (v) Normal rats treated with thymol. The rats were injected with STZ at a dose of 55 mg/kg body weight intraperitoneally to induce diabetes. Thymol was given orally at a dose of 40mg/kg body weight for 28 days. At the end of the study, the rats were sacrificed and blood samples were drawn by cardiac puncture to determine the levels of Blood glucose, Plasma Alkaline phosphatase (ALP), Total Cholesterol, Triglycerides, Low-density lipoprotein (LDL), High-density lipoprotein (HDL), Urea, Creatinine, Serum glutamic-oxaloacetic transaminase (SGOT), Serum glutamate pyruvate transaminase (SGPT), Plasma malondialdehyde (MDA) content, Advanced oxidation protein products (AOPP), Sialic acid (SA). Markers of antioxidants as Ferric reducing antioxidant power (FRAP) and erythrocyte Reduced Glutathione (GSH) were also measured. Radical scavenging activity in terms of percent 2, 2-diphenyl -1- picrylhydrazyl (DPPH). Blood glucose level, Total Cholesterol, Triglycerides, LDL, Urea, Creatinine, SGOT, SGPT, ALP, MDA, AOPP, SA levels which were found elevated due to diabetes reduced significantly to near normal. However, HDL level, % DPPH, FRAP content and erythrocyte GSH levels elevated after supplementation. We observe that administration of thymol afforded remarkable protection against diabetes and its related complications.

Key words: Thymol, *Nigella sativa,* Streptozotocin, Cholesterol.

Introduction

Diabetes mellitus is one of the most common disorders affecting almost 8.8% of the world's population. According to International Diabetes Federation (IDF) estimates there are 424.9 million adults (aged 20-79) worldwide with diabetes mellitus (DM), a number that is expected to reach to 628.6 million by 2045 (IDF, 2017).

Globally, diabetes is one of the six major causes of death (Petchi *et al.,* 2014). Medical interventions to manage diabetes include glucose-lowering agents such as alpha-glucosidase inhibitors, biguanides, sulfonylureas and thiazolidinediones and sometimes insulin. All these interventions are associated with severe to mild side effects after a given amount of time. These compulsions provide a perfect setting for use of alternative systems of medicine based on traditional knowledge (Parasuraman *et al.,* 2010).

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Plants and their active compounds have frequently been used for medicinal purposes. According to the World Health Organization (WHO), "a medicinal plant is a plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes, or which are precursors for chemo-pharmaceutical semi-synthesis." Such plants are in great demand by pharmaceutical companies for their active ingredients (Huai *et al.,* 2010, Husain *et al.,* 2008).

In the Indian traditional system of medicine, *Nigella sativa*, an annual herb belonging to Ranunculaceae family have been used for thousands of years as a spice and food preservative to a variety of food products as bread, yogurt, pickles, sauces, salads etc. (Hajhashemi *et al.,* 2004). *Nigella sativa* is traditionally used for its galactagogue, appetizer, thermogenic and diuretic effects (Hosseinzadeh *et al.,* 2013). Furthermore, *Nigella sativa* possesses anti-microbial, anti-fungal, anti-oxidative and

anti-cancerous properties (Salem and Hossain, 2000, Salomi *et al.,* 1992). *Nigella sativa* seeds contain several bioactive compounds, one of them is thymol (THY).

Thymol (2-isopropyl-5-methylphenol) is a dietary monoterpene phenol exhibiting multiple biological activities including antioxidant (Aeschbach *et al.,* 1994) and free radical scavenging properties (Fujisawa *et al.,* 1992). The present study is undertaken to investigate the anti-diabetic potential of thymol on STZ-induced diabetes in rats.

Materials and Methods

Reagents and chemicals

Thymol was procured from Sigma Aldrich, India. All other chemicals were of analytical grade and made available from Merck, India and Himedia Labs, India.

Experimental animals and STZ induction

The experiment was carried out with 30 male Wistar rats (5 ± 0.5 months and body weight 150 ± 20 g). They were housed in a temperature controlled room (25 ± 5°C) with12-h. light-dark cycles for at least 1 week. All rats were fed with a normal laboratory diet of nutrient rich pellets containing total energy as fat, protein and carbohydrates and had free access to drinking water throughout the period of the experiment.

Experimental design

Rats were divided into five groups of six rats each and were given the following treatments:

Group I: Normal Control (NC) receiving no treatment.

Group II: Diabetic Control (DC), rats were injected single dose of STZ (55 mg/kg body weight) intra-peritoneally.

Group III: STZ induced diabetic rats were supplemented with insulin (D+INS).

Group IV: STZ induced diabetic rats were supplemented with thymol via gavage technique (oral route) at 40mg/kg body weight/day for 28 days (D+THY).

Group V: Normal rats were given only thymol (40 mg/kg body weight/day for 28 days) (N+THY).

Diabetes was induced by intra-peritoneal injection of STZ at a dose of 55 mg/kg body weight. After 96 hours diabetes was confirmed by the determination of fasting blood glucose level with the help of a glucometer, rats with blood glucose (>250 mg/dl) were considered diabetic and included in the study. All treatment was carried out up to 28 days. The animals of the first group were simultaneously administered water until 28 days.

At the end of the experimental period, the rats were fasted for overnight (12 hours) and sacrificed under light anaesthesia. Blood samples were collected by cardiac puncture into 10 unit/ml heparin rinsed anticoagulant syringes. Plasma was obtained from blood sample after centrifugation (1500×g for 10 min) and stored at 4°C for analysis. After the removal of plasma (immediately frozen at -80°C until use for biochemical assays), buffy coat and the upper 15% of packed red blood cells (PRBCs), the RBCs were washed twice with cold phosphate buffered saline (PBS) (0.9% NaCl and 10 mmol/l Na_2HPO_4, pH 7.4) and then used for experiment. All protocols for experiments were approved by the Animal Care and Ethics Committee of University of Allahabad.

Biochemical assays

Blood glucose values were determined using an Accu-Check Active Glucometer (Roche Diagnostics, Mannheim, Germany). Lipid profile (Total Cholesterol, HDL, LDL), SGOT, SGPT, urea, creatinine and alkaline phosphatase were measured using reagent kits from Erba Diagnostics, Mannheim, Germany.

Measurement of total antioxidant activity by FRAP

The total antioxidant potential of the plasma samples was determined using a modification of the ferric reducing ability of plasma (FRAP) assay of Benzie and Strain (1996). FRAP reagent was prepared from 300 µmol/l acetate buffer, pH 3.6, 20 µmol/l ferric chloride and 10 mmol/l TPTZ made up in 40 µmol/l hydrochloric acid. All three solutions were mixed together in the ratio 10:1:1 (v:v:v) respectively, 3 ml of FRAP reagent was mixed with 100 µl of plasma and the contents were mixed thoroughly. The absorbance was read at 593 nm at 30 s intervals for 4 min. Aqueous solution of known Fe(II) concentration in the range of 100-1000 µmol/l was used for calibration. Regression equation of the FRAP values (µmol Fe(II)/l) of the plasma was used for calculation.

Determination of DPPH radical scavenging activity in plasma

This assay was performed according to the method proposed by Szabo *et al.,* (2007). 100 µl of plasma was added to 10 mmol phosphate buffer (1.9 ml) and 0.1 mmol 2, 2-diphenyl-1-picrylhydrazyl (DPPH) in methanol (2.0 ml) with a control having 2 ml of 10 mmol phosphate buffer with the same amount of DPPH solution. It was kept for incubation for 30 min at 21°C and centrifuged for 5 min at 1000×g. Absorbance was measured at 517 nm with methanol as a blank. Values were compared for control (A_0) and plasma (A) and percent radical scavenging activity was calculated by using 100 $(A_0 - A)/A_0$.

Determination of erythrocyte reduced glutathione (GSH)

Erythrocyte GSH was measured following the method of Beutler (1984). The method is based on the ability of the SH group to reduce DTNB and form a yellow coloured anionic product whose optical density is

measured at 412 nm. Concentration of GSH is expressed in mg/ml packed RBCs.

Determination of plasma advanced oxidation protein products (AOPPs)

Determination of AOPPs in plasma was based on spectrophotometric detection according to Witko-Sarsat *et al.,* (1996). Briefly, 200 ml of plasma (diluted 1:5 with phosphate buffer saline (PBS) as test), 200 ml of chloramine-T solution (0-100) µmol/l for calibration and 200 ml of PBS as blank were applied. 10 µl of 1.16 kol potassium iodide and 20 µl of acetic acid were added and absorbance at 340 nm was measured immediately. Concentration of AOPPs was expressed as µmol/l of chloramine-T equivalents.

Determination of plasma Sialic acid (SA) level

Sialic acid was determined by the method of Spyridaki *et al.,* (1996). Briefly, 0.5 ml of plasma was treated with 0.10 ml of 0.04 mol periodic acid. It was mixed thoroughly and allowed to stand in ice bath for 30 min. Thereafter 1.25 ml of resorcinol working solution (5 ml of 6.0% resorcinol solution, 0.125 ml of 0.1 mol copper sulphate solution and 19.875 ml of distilled water, brought to a final volume of 50 ml with 10 mol HCl) was added, mixed and heated at 98°C for 5 min. Thereafter cooled in an ice bath for approximately 2 min. Lastly 3.25 ml of n-butanol was added and mixed well. Placed in a water bath at 37°C for 3 min for stabilizing the colour. Their absorbance was measured at 625nm against a reagent blank. Plasma Sialic acid is measured as µmol.

Determination of lipid peroxidation (malondialdehyde (MDA) level) in plasma and erythrocytes

Plasma or erythrocyte MDA was measured according to the method of Esterbauer and Cheeseman (1990) with slight modification. Packed RBC (0.2 ml) was suspended in 3 ml PBS containing 0.5 mmol/l glucose, pH 7.4. The lysate (0.2 ml) was added to 1 ml of 10% trichloroacetic acid (TCA) and 2 ml of 0.67% thiobarbituric acid (TBA) boiled for 20 min at 90–100°C, cooled and then the mixture was centrifuged at 1000×g for 5 min and the absorbance of supernatant was read at 532 nm. The concentration of MDA in erythrocytes was calculated using extinction coefficient ($\varepsilon = 31,500$) and is expressed as nmol/ml of packed RBC.

Statistical analysis

Statistical analysis was performed using one-way ANOVA followed by Tukey's Multiple Comparison Test with Graph Pad PRISM Software, San Diego, CA, USA, version 5.01 for Windows. All values are expressed as the mean ± SD. All the values with $P<0.05$ were considered as statistically significant.

Results and Discussion

Diabetes mellitus is a pathologic condition, resulting in severe metabolic imbalance and non-physiologic changes in many tissues more particularly in pancreas, where oxidative stress plays an important role in the etiology (Baynes and Thorpe, 1996). Diabetics and experimental animal models exhibit high oxidative stress due to persistent and chronic hyperglycemia which promotes free radical generation (Baynes and Thorpe, 1996). In diabetes mellitus, alterations in the endogenous free radical scavenging defence mechanisms may lead to ineffective scavenging of reactive oxygen species, resulting in oxidative damage and tissue injury. Ihara *et al.,* (1999) examined oxidative stress markers in experimental diabetic rats and found increased reactive oxygen species (ROS) in pancreatic islets. Free radicals may play an important role in the development of late complications of diabetes mellitus (Mohamed, 1999).

In this study, the anti-hyperglycemic effect of the thymol supplementation was investigated using STZ induced diabetic rats. After 28 days of oral administration of thymol, significant reduction in blood glucose levels was observed in the STZ-induced diabetic rats. The blood glucose was significantly elevated ($P<0.01$) in diabetic rats as compared to normal control rats (fig. 1). In diabetic rats, oral administration of thymol (40 mg/kg body weight) lowered the blood glucose significantly ($P<0.01$). Our findings agree with the findings of Saravanan and Pari (2015), who observed that thymol treated group showed significantly decreased blood glucose levels (Fig. 1).

The lipid profiles and hepatic parameters of control and treated rats were evaluated (Table 1). Table 1, shows an increase in levels of Total cholesterol (56%), Triglyceride (52%) and LDL-Cholesterol (199%) as well as a decrease in HDL-Cholesterol (31%) of diabetic rats as compared to the control group. In addition, data also showed a significant ($p<0.05$) increase in the activities of SGOT (61%), SGPT (240%) and ALP (116%) in diabetic rats as compared to the control rats, suggesting hepatocellular damage as a result of STZ toxicity. These observed increased levels in diabetic rats may be explained due to oxidative damage caused by the diabetogenic agent STZ. It was also suggested that hepatic damage may cause this abnormal rise in lipid profile parameters and liver enzymes levels (Asante *et al.,* 2016). Interestingly, the thymol administration showed an important protective action in diabetic rats by reducing the hepatic toxicity. Indeed, thymol treatment significantly reduced the SGOT ($p<0.05$), SGPT ($p<0.01$) and ALP ($p<0.001$) enzyme activities and the obtained values were nearly similar to those of the control rats. Furthermore, as shown in table

Table 1: Effect of Thymol supplementation on biochemical profile of STZ-induced diabetic rats.

S. No.	Parameters	Normal Control	Diabetic Control	Diabetes + Insulin	Diabetes + Thymol	Normal + Thymol
1.	Total Cholesterol (mg/dl)	93±7	145 ± 16***[a]	119± 8***[b]	120 ± 8***[b]	90 ± 5
2.	HDL-C (mg/dl)	48 ± 5	33 ± 7**[a]	35± 8[b]	37 ± 8[b]	44 ± 4
3.	LDL-C (mg/dl)	40.36 ± 8	120.53±9***[a]	87.55± 9***[b]	80.55 ± 9***[b]	33.50 ± 6
4.	Triglyceride (mg/dl)	89 ± 7	135 ± 15***[a]	107± 9***[b]	107 ± 9***[b]	85 ± 11
5.	SGOT (U/L)	105 ± 9	169 ± 5***[a]	137± 12*[b]	132 ± 6*[b]	111 ± 7
6.	SGPT (U/L)	5.59 ± 1.49	19.03±.07***[a]	11.47±2.2***[b]	13.47 ± 2.21**[b]	4.47 ± 0.92
7.	Alkaline Phosphatase (U/L)	252.56± 9.12	545 ± 37.27***[a]	349± 30***[b]	392 ± 30.21***[b]	261 ± 24.59
8.	Urea (mg/dl)	39.90 ± 4.38	61.24 ± 7.34***[a]	52± 5.89[b]	50 ± 5.89*[b]	43 ± 4.03
9.	Creatinine (mg/dl)	0.65 ± 0.08	1.34 ± 0.218***[a]	0.82± 0.09***[b]	0.67 ± 0.09***[b]	0.59 ± 0.03

Values represent mean ±SD for 6 rats. *p < 0.05, **p < 0.01, ***p < 0.001.
[a]Diabetic group as compared to control group, [b]Experimental group as compared to Diabetic control.

1, the plasma lipid profile progressed to the normal values of control rats which are in agreement with reported findings (Asante *et al.,* 2016). It is also important to note that thymol effect on the hepatic dysfunction parameters and lipid profile was similar to that obtained by standard insulin (Table 1).

Table 1, presents the kidney toxicity indices of control and treated rats. The obtained results showed that hyperglycemia increased the urea and creatinine levels by 53 and 106%, respectively in diabetic rats as compared to control group. Thymol administration to diabetic rats improved the indices related to kidney dysfunction induced by diabetes. In fact, thymol administration to diabetic rats significantly decreased the urea ($p<0.05$) and creatinine ($p<0.001$) levels by 10% and 38%, respectively, which is similar to other studies (Jdir *et al.,* 2017). As compared to normal individuals, high urea and creatinine levels represent important kidney dysfunction markers (Liu *et al.,* 2006). Thus, it can be concluded that diabetic rats suffer from renal disorders due to the protein glycation that lead to the muscle loss as well as to the increase of purine release, which is the main uric acid source (Jagdale *et al.,* 2016). The alleviation of kidney dysfunction parameters could be explained by the attenuation of oxidative stress situation *via* glucose level regulation.

Oxidative stress is an imbalance between reactive oxygen species (ROS) production and the antioxidant defence systems (Favier, 1997). The measure of plasma anti-oxidant capacity revealed a good association of oxidative stress in several pathologies and particularly, in the diabetes mellitus (Bonnefont-Rousselot *et al.,* 2000). To combat ROS, the organism has enzymatic defence mechanisms as superoxide dismutase, glutathione peroxidase or catalase and non-enzymatic types as glutathione and α-tocopherol. The ROS scavenging capacity through the antioxidant systems becomes insufficient in diabetes and a constant oxidative stress develops (Ohkawa *et al.,* 1979). Due to the large number of antioxidants present in plasma, several methods have been developed: ferric reducing antioxidant power (FRAP), DPPH radical scavenging activity and Reduced glutathione (GSH) level (Janaszewska and Bartosz, 2002, Huang *et al.,* 2005).

In our study, plasma FRAP value significantly depleted ($p<0.001$) in STZ-induced diabetic control rats as compared to normal control rats. After thymol supplementation, FRAP value significantly ($p<0.001$) improved in STZ-induced diabetic rats as compared to diabetic control rats (Fig. 2). These findings are in concordance with the study of Sasvari and Nyakas (2003). Similar results were found with percent radical scavenging activity (Fig. 3) and reduced glutathione level (Fig. 4) in our study as well as studies of Meziti *et al.,* (2012).

Reduced radical scavenging activity of plasma causes oxidative modulation of proteins which may be one of the reasons of altered physiological processes in type 2 diabetic patients (Pandey *et al.,* 2010). Therefore, the measurement of the protein oxidation is a clinically important factor for the prediction of the diabetes or degree of oxidative stress in diabetes and stress-related diseases. Advanced protein oxidation products (AOPP) are defined as dityrosine containing crosslinked protein products due to action of chloraminated oxidants, mainly hypochlorous acid and chloramines, produced by myeloperoxidase in activated neutrophils. It is considered a reliable marker for estimating the degree of protein oxidative modification (Witko-Sarsat *et al.,* 1996). Oxidation of proteins can lead to a whole variety of amino acid modifications, it may be selective and specific. Accumulation of protein products is associated with a number of diseases, including coronary artery diseases (Kaneda *et al.,* 2002), diabetes (Martin-Gallan *et al.,* 2003), preterm neonates (Buonocore *et al.,* 2002) and dentritic cell stimulation (Witko-Sarsat *et al.,* 1998). In our study, a significant ($P<0.001$) elevation in AOPP level

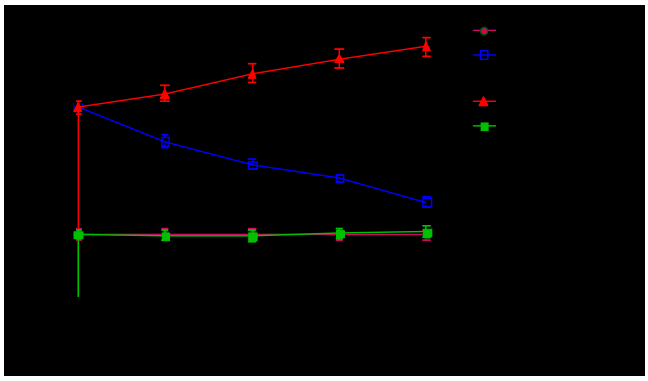

Fig. 1: Effect of Thymol Supplementation on Blood Glucose Level of STZ- induced Diabetic Rats.

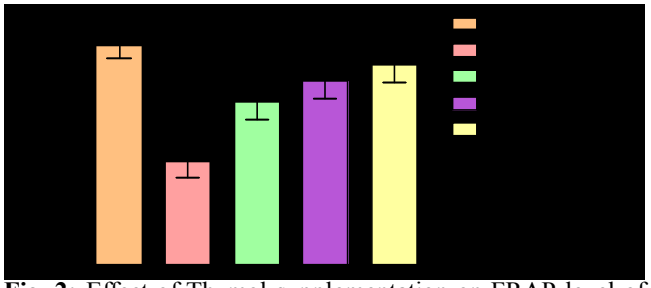

Fig. 2: Effect of Thymol supplementation on FRAP level of STZ-induced diabetic rats. FRAP value is expressed in µmolFe(II)/l plasma. Values represent mean ±SD. ***($p < 0.001$) as compared to Diabetic control.

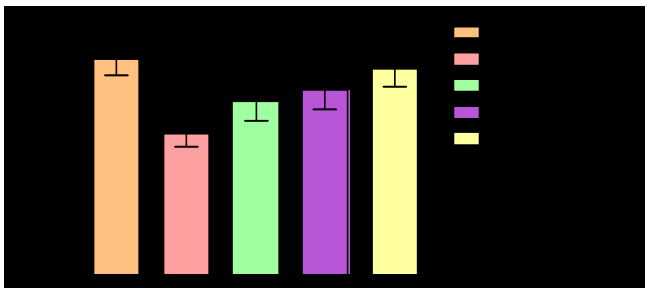

Fig. 3: Effect of Thymol supplementation on DPPH % inhibition of STZ-induced diabetic rats. Values represent mean ±SD. ***($p < 0.001$) as compared to normal control. **($p < 0.01$) as compared to diabetic control. *($p < 0.05$) as compared to diabetic control.

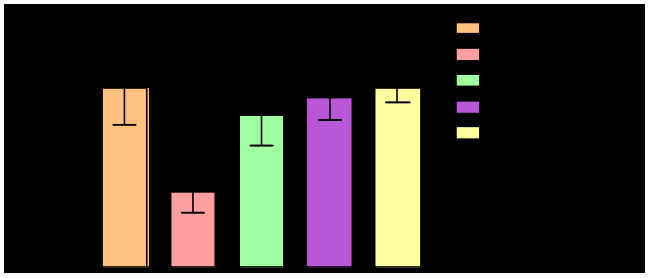

Fig. 4: Effect of Thymol supplementation on Reduced glutathione (GSH) level of STZ-induced diabetic rats. GSH value is expressed in mg/ml of packed erythrocytes. Values represent mean ±SD. ***($p < 0.001$) as compared to diabetic control.

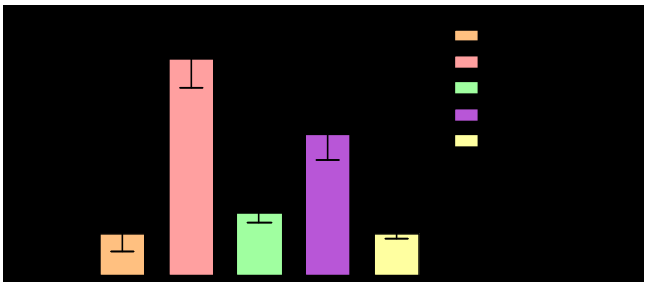

Fig. 5: Effect of Thymol supplementation on AOPP level of STZ-induced diabetic rats. AOPP value is expressed in µmol/l of plasma. Values represent mean ±SD. ***($p < 0.001$) as compared to diabetic control.

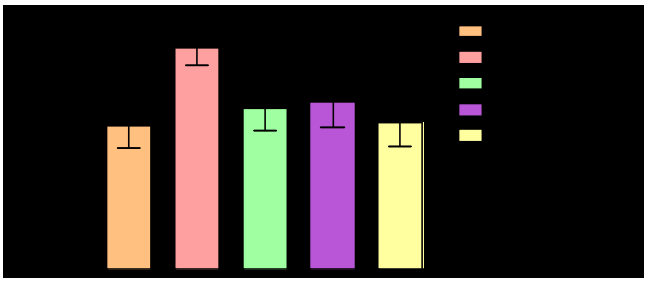

Fig. 6: Effect of Thymol supplementation on Sialic acid content of STZ-induced diabetic rats. Sialic acid value is expressed in µmol of plasma. Values represent mean ±SD. ***($p < 0.001$) as compared to diabetic control. **($p < 0.01$) as compared to diabetic control.

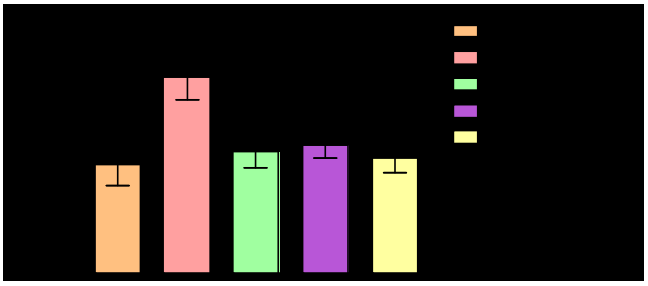

Fig. 7: Effect of Thymol supplementation on plasma malondialdehyde (MDA) content of STZ-induced diabetic rats. Concentration of MDA is expressed as nmol/ml of plasma. Values represent mean ±SD. ***($p < 0.001$) as compared to diabetic control.

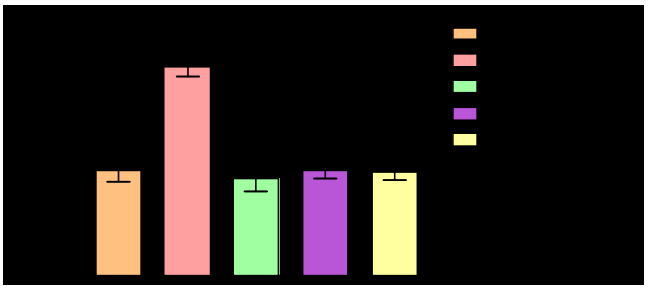

Fig. 8: Effect of Thymol supplementation on erythrocyte malondialdehyde (MDA) content of STZ-induced diabetic rats. Concentration of MDA is expressed as nmol/ml of packed erythrocytes. Values represent mean ± SD. ***($p < 0.001$) as compared to diabetic control.

was observed in diabetic group as compared to control, which significantly (P<0.01) decreased after the administration of thymol (Fig. 5). This study showed that thymol is as effective as insulin in decreasing the levels of AOPP value and blood glucose level in STZ induced diabetic rats.

Sialic Acid (SA) is an acetylated derivative of neuraminic acid and is an essential component of glycoproteins and glycolipids. Vascular endothelium carries a high concentration of Sialic acid where it governs permeability. It is necessary for the cell-surface residency of platelet and promotes endothelial barrier integrity (Cioffi *et al.,* 2012). It also acts as a co-factor of many cell receptors and is positively associated with most of the serum acute phase reactants. In diabetic state, extensive micro vascular damage sheds Sialic acid into circulation (Prakash and Sudha, 2013). Several studies have highlighted that Sialic acid metabolism is drastically altered in diabetic condition. Such an elevation of Sialic acid level in the plasma leads to complications like retinopathy, nephropathy and neuropathy. A study of Prajna *et al.,* (2013) states that increased SA is a potential risk factor for development of nephropathy in diabetic patients. Similarly raised levels of serum Sialic acid are implicated in cardiovascular diseases. In our study, a significant elevation (P<0.001) in plasma Sialic acid was observed in diabetic control rat. Oral administration of thymol to STZ induced diabetic rat restored the levels of Sialic acid in plasma to near normal (Fig. 6).

Lipid peroxidation (LPO) is a well-established mechanism of cellular injury and is used as an indicator of oxidative stress in cells and tissues. Measurement of malondialdehyde has been used as an indicator of lipid peroxidation. LPO is the process of oxidative degradation of polyunsaturated fatty acids and its occurrence in biological membranes causes impaired membrane function, structural integrity, decrease in membrane fluidity and inactivation of several membrane bound enzymes (Pandey and Rizvi, 2010). Under oxidative stress, the erythrocyte membrane is prone to lipid peroxidation that involves cleavage of polyunsaturated fatty acids at their double bonds, leading to the formation of MDA, an increased MDA content is an important indicator of lipid peroxidation. Increased level of MDA in erythrocyte has been reported in many disease conditions which are accompanied with oxidative stress (Bhatia *et al.,* 2003). In this study, plasma and Erythrocytes MDA content was significantly (p<0.001) increased in the STZ induced diabetic rats as compared to normal control rats and in contrast, MDA level was significantly decreased (p<0.001) in diabetic rats when supplemented with thymol (Fig. 7 and 8). This toxicity may be due to the alterations in membrane integrity *via* the formation of reactive oxygen species by successive hydroperoxide formation and β cleavage of polyunsaturated fatty acids *in vivo* or due to perturbation of antioxidant defence mechanisms.

Conclusion

Results obtained in the present study suggest that thymol, an active constituent of *Nigella sativa* has an overall protective effect against STZ induced diabetes mellitus in a rat model. Thymol produces significant anti-hyperglycaemic effect in diabetic rats. We suggest that these results provide strong evidence in support of the pharmacological use of thymol as an anti-diabetic complement in cases of type 2 diabetes.

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