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Natural antioxidants from plant products have been reported to possess free radical scavenging properties and prevent oxidative damage without causing unwanted effects. Among various classes of chemicals present in the plants, phenolic compounds have tremendous health promoting effects as antioxidants. The phenolic compounds exhibit considerable free radical scavenging activities through their electron-donating and metal ion chelating properties. Since some antioxidants may act as inhibitors of glycation, in the first phase of study, we evaluated in-vitro antioxidant activities of three phytochemicals: thymoquinone (TQ), thymol (TL) and eugenol (EU) by determining reducing power, ferric reducing antioxidant power (FRAP), metal chelating activity, DPPH and AAPH radical scavenging assays. The phytochemicals were also found to have properties of chelating ferrous ions. Based on these data, it is clear that the TQ, TL and EU contained high antioxidant activity and chelating properties. The all three phytochemicals were found to possess strong radical-scavenging and redox abilities as evidenced by model antioxidant assays including DPPH and FRAP. To evaluate whether the tested phytochemicals could affect glycation induced generation of ROS, cytochrome c reduction assay was employed. A significant decrease in the generation of superoxide radical was observed in the presence of these phytochemicals. This seemingly indicates that the phytochemicals either scavenge superoxide anion radicals or chelate transition metals leading to less free radical production, or they may have both effects. The protective effect of these phytochemicals on AAPH-induced RBC hemolysis was observed in a dose dependent manner. EU possesses lower IC$_{50}$ value as compared to TL and EU contained high antioxidant activity and chelating properties. The all three phytochemicals were found to possess strong radical-scavenging and redox abilities as evidenced by model antioxidant assays including DPPH and FRAP. To evaluate whether the tested phytochemicals could affect glycation induced generation of ROS, cytochrome c reduction assay was employed. A significant decrease in the generation of superoxide radical was observed in the presence of these phytochemicals. This seemingly indicates that the phytochemicals either scavenge superoxide anion radicals or chelate transition metals leading to less free radical production, or they may have both effects. The protective effect of these phytochemicals on AAPH-induced RBC hemolysis was observed in a dose dependent manner. EU possesses lower IC$_{50}$ value as compared to TL and EU contained high antioxidant activity and chelating properties. Among three phytochemicals, EU had the most powerful antioxidant and radical scavenging activity followed by TL and TQ which is related to their structure. TQ has weak electron donating groups contributing to less electron density to the benzene ring. However, electron density is found to be higher in TL and EU because of presence of hydroxyl group attached to benzene ring. Among EU and TL, EU has two electron donating groups viz. methoxy group (—OCH$_3$) and allyl group (—CH$_2$—CH=CH$_2$) at ortho- and para- position respectively which are more reactive sites as compared to meta- position while TL has weak electron donating groups at ortho- and meta- position (methyl and isopropyl) respectively. Hence it can be concluded that EU having maximum electron density showed best antioxidant activity among the three phytochemicals.

Non-enzymatic glycation, the reaction of glucose and other reducing sugars with amino group of proteins, produces Amadori or early glycation products, while longer exposure results in irreversible advanced glycation end products (AGEs). Glycation involves non-enzymatic covalent attachment of carbonyl groups of glucose with N-terminal and lysyl side chain e-amino groups to form unstable Schiff base adduct that rapidly progresses to a...
stable ketoamine derivative, the Amadori product. Hereafter, the reactions become more varied and complicated leading to the formation of AGEs. AGE formation is greatly accelerated in hyperglycaemic conditions and many studies so far have demonstrated the formation and role of AGEs in various diseases including diabetes. Glycation alters protein conformation and induces protein cross linking that eventually ensues in aggregation. Modifications of structural as well as circulatory proteins by glycation have drawn much attention because of their potential role in the etiopathogenesis of various diseases.

As the glycation is a dose and time dependent process, in the second phase, a comparative study of HSA denaturation/degradation induced by glucose for extended time period was performed. The structural perturbations in the glycated HSA samples were analyzed by UV absorbance, tryptophan fluorescence, circular dichroism, FTIR and gel electrophoresis techniques. The studies revealed remarkable structural and biophysical changes in HSA upon glycation by glucose up to 28 days. Estimation of ketoamine, carbonyl and free amino groups revealed that glycation induces conformational and structural changes in HSA. UV spectra of glycated HSA showed hyperchromicity as observed on day 14 and post incubation hypochromicity thereafter. These changes are indicative of positional change of aromatic acids of HSA upon its glycation. The tryptophan fluorescence of HSA showed the same pattern as observed in UV spectra presumably involving same aromatic amino acids are involved in both spectral analysis. Glycation causes unfolding of protein leading to exposure of aromatic amino acids towards solvent system resulting in hyperchromicity and gain in tryptophan fluorescence. However, on further incubation up to 28 days causes shielding of aromatic amino acids contributing to hypochromicity and loss of fluorescence intensity. The significant observation in the tryptophan fluorescence was a blue shift on glycation. The results reiterate the earlier observation and suggest conformational changes in glycated HSA. The fluorescence intensity of AGEs was also found to be increased in glycated HSA samples compared to native HSA.

The Far-UV CD spectrum of glycated HSA showed a loss of helical structure as shown by a decrease in the negative ellipticity at 208 and 222 nm. The interaction between native and glycated HSA was confirmed by FTIR spectral analysis as well. Glycation of protein has been shown to result in the protein degradation and/or cross-linking and the observed alterations in the electrophoretic behavior of HSA incubated with glucose apparently results from such effects. Glycated HSA migrated as highly diffuse band with the increase in incubation time and showed the presence of aggregates. However, native HSA migrated as a single band of 66kD molecular weight. Next, colorimetric estimations were carried out to support the biophysical analysis. Ketoamine level at 7 days of incubation was found to be significantly higher in glycated HSA as compared to native HSA, which showed negligible ketoamine content. Levels of carbonyl groups were also
Abstract

Elevated in glycated HSA, an important marker of both glycation and oxidative stress. Number of free amino groups in glycated HSA samples was found to be decreased as compared to native HSA. On the basis of above observations we can infer that incubations with glucose for up to 28 days resulted in a time dependent modification of HSA. Thus, prolonged exposure of HSA to glucose exerts greater deleterious effects on its structure and formation of aggregates.

Hyperglycaemia and accumulation of AGEs due to non-enzymatic glycation of proteins in tissues and serum have important roles in diabetic complications. Moreover, Amadori product is the principal form of glycation mediated modification in proteins. Recent investigations have shown that elevated concentrations of Amadori products play a substantial role in diabetes-related complications. Although there have been important advances in the control of the hyperglycaemia of diabetes by means of diet, hypoglycaemic drugs, insulin and islet transplantation. The long term complications of diabetes such as cataract, nephropathy, retinopathy and atherosclerosis are still leading causes of death. These complications are a direct result of protein alteration which results in irreversible tissue damage. Thus, inhibition of the formation of AGE is believed to play a role in the prevention of diabetes-related complications. Designing a drug having anti-AGE activity is a challenge due to the complexity of reaction involved in the formation of AGE. The most studied and successful agent has been a nucleophilic hydrazine compound viz. aminoguanidine (AG) has shown promising results in-vitro and in animal models in terms of inhibition of AGE formation. A number of other agents such as pyridoxamine, carnosine, taurine and phenyl thiazolium bromide have also been investigated in several studies and have shown promising results. However, except pyridoxamine, none has progressed as yet to the stage of clinical trials. Although some recent studies highlighted the antiglycating potential of a few natural sources, namely garlic, green tea and tomato adequate work has not yet been done. Thus there is a need for developing new antiglycating agents combining higher levels of efficacy, selectivity and safety in humans. Therefore, the identification of antiglycation compounds is attracting considerable interest. Many dietary agents, particularly spices, are a major part of traditional medicine that has been practiced to control many chronic ailments including diabetes.

TQ, TL and EU are commonly used commodities of diet and/or traditional medicine. Therefore, inhibitory effect of varying concentrations of TQ, TL and EU on glycation to HSA induced by glucose was evaluated up to 28 days in-vitro. The interaction of HSA with glucose in presence of these phytochemicals was studied by absorbance, fluorescence and FTIR techniques. Both hyperchromicity and hypochromicity was found to be decreased in presence of aminoguanidine (AG) and at all concentrations of TQ, TL and EU. Eugenol (0.6 µM) showed highest reduction in hyper and hypochromicity in comparison to other formulations of its group. The reduction in conformational changes
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due to glycation in presence of AG and phytochemicals were evident at all time points. It indicates that the TQ, TL and EU inhibit the glycation of HSA as observed by a reduction in the hyperchromicity at 7 and 14 days and decrease in hypochromicity at 21 and 28 days. Fluorescence spectra of HSA also exhibited the same pattern as observed in UV spectra. The characterization study of AGEs was performed using AGE-specific fluorescence and quantitation by free lysine side chains. Our results indicated that these phytochemicals at all concentrations inhibit the glycation of HSA as observed by a reduction in the formation of fluorescent AGEs at 14, 21 and 28 days of incubation. Further, formation of new peaks on addition of AG, TQ, TL or EU in the infra-red region confirms the interaction of these phytochemicals with HSA as observed by FTIR spectra.

Ketoamines are early non-enzymatic glycation adducts and are important precursors of AGEs and hydroxyl radicals. Inhibition of ketoamine formation in glycated HSA in presence of AG or varying concentrations of TQ, TL and EU was observed significantly at 7 days. Middle concentration of EU tested i.e. 0.6 µM showed maximum inhibition of ketoamine formation hence, helpful in reducing the formation of glycation induced intermediary compounds. Ketoamines were converted to protein bound carbonyl groups via a protein enediel reaction. The generation of carbonyl groups serves as a marker of protein glycoxidation. The presence of AG and these phytochemicals in glycated HSA significantly reduce the level of carbonyl content at 7, 14, 21 and 28 days. Further these phytochemicals reduced the amount of modified lysine side chains as compared to the control. Free radical generation during glycation was confirmed by quantitation of superoxide radicals in presence and absence of AG, TQ, TL, EU and SOD. The results indicate that early glycation generates free radicals which were quenched significantly by these phytochemicals and AG. Studies with these phytochemicals showed inhibition of different parameters in glycated HSA samples showing a definite role of ROS in the modification of glycated HSA and AGE formation.

Recent evidences suggest that increased oxidative damage as well as reduction in antioxidant capacity could be related to the complications in patients with type-2 diabetes. Thus, the study was extended further to evaluate the antioxidative and antiglycative role of TQ (30 µM), TL (30 µM) and EU (0.6 µM) in diabetic patients with secondary complications. We observed the changes in MDA, protein carbonyl, FRAP, glutathione (GSH) levels, protein crosslinking and/or fragmentation in sera of these patients incubated in presence and absence of these phytochemicals for 21 days. Sera of healthy individual without any treatment served as control. Malondialdehyde (MDA) and protein carbonyl levels were evaluated to determine the lipid and protein damage in serum. FRAP value and glutathione level was taken as the indicator of total antioxidation potential. MDA and carbonyl content was found significantly increased which strongly supported the increased oxidative damage in case of diabetic patients as compared to healthy subjects. In-vitro treatment with these phytochemicals showed a significant
decrease in their level in type-2 diabetic patients. The level of reduced glutathione was significantly lower in the type-2 diabetic patients as compared to normal subjects. On the in-vitro treatment with phytochemicals there was increase in its level in type-2 diabetic patients. FRAP value in serum was also lowered significantly in type-2 diabetic patients. Phytochemical treatment ameliorates FRAP value. Decrease in MDA and carbonyl content with a concomitant increase in GSH and FRAP levels on in-vitro treatment with phytochemicals were found to be more pronounced in the case of eugenol at 0.6 µM followed by thymol (30 µM) and thymoquinone (30 µM).

Serum profile of diabetic patient shows extensive HSA cross-linking, fragmentation and aggregate formation as observed by diffusion of band but the sera incubated with TQ, TL and EU exhibited inhibition of HSA cross-linking, fragmentation and aggregate formation. Amongst the three phytochemicals, EU at the 0.6 µM concentration could inhibit diffusion of band better than 30 µM of TQ and TL. The intake of these phytochemicals may be helpful in diabetes related complications.

All the three phytochemicals described in the present study have cumulative effect of antioxidant and antiglycation activities that might contribute to effective action. However, the in-vitro results may not reflect the effects of these agents in-vivo as they undergo biotransformation process followed by the liver first pass effect, which invariably affect the content, activity and bioavailability of these compounds. Hence, further investigations are needed to address these issues. Various substances included in the present study are naturally occurring. This fact and the results of the present study indicate the possibility of therapeutic use of these phytochemicals for the prevention of diabetic complications. An important therapeutic factor worth of consideration is to administer the phytochemical to diabetic patients. This is essential because it has been proposed that once the progress of excessive glycation has begun, subsequent remediation of hyperglycaemia would not prevent diabetes related complications.
Introduction
Glycation is a non-enzymatic reaction of the amino groups of amino acids, peptides, and proteins with carbonyl group of reducing sugars resulting in the formation of complex brown pigments and protein-protein crosslinks. It is occurring slowly but continuously in cells of all living organisms. It was first studied under defined conditions by Louis Camille Maillard in the early 1900s (Maillard and Gautier, 1912). Thus it came to be known as the Maillard reaction (Fig. 1). Exact realization of the importance of Maillard-like reactions \textit{in-vivo} began in the mid-1970s when studies was done on haemoglobin A\textsubscript{1c} (HbA\textsubscript{1c}), a naturally occurring minor human haemoglobin that is elevated in diabetics (Bookchin and Gallop, 1968). HbA\textsubscript{1c} was known to be a post-translational adduct of glucose with the N-terminal valine amino group of the \(\beta\) chain of hemoglobin, in which the glucose was thought to be attached via non-enzymatically formed Schiff base structure. We found that measurement of the elevation of HbA\textsubscript{1c} in diabetics allowed assessment of the degree of glucose control integrated over several weeks (Koenig \textit{et al.}, 1976). Later on, the significance of the complex, late stage Maillard processes was recognized as mediators of several complications in diabetes (Bunn \textit{et al.}, 1978) and aging (Monnier and Cerami, 1981). The Maillard reaction is actually a complex series of reactions and is sub-divided into three main stages: early, intermediate, and late.

\textit{Early Stage:}

The nucleophilic addition reaction between a carbonyl group from a reducing sugar (e.g. glucose, fructose, galactose, mannose etc) and a free amino group is initiated with the reversible formation of an adduct known as Schiff base by conversion of the aldehydic carbon–oxygen double bond of the sugar to a carbon–nitrogen double bond with the amine. This reaction occurs over a period of hours. The Schiff base is a thermodynamically unstable form in relation to the equilibrium cycled pyranose or furanose forms. Therefore, the Schiff base gives rise to an enaminol intermediate by rearrangement and, subsequently, to a relatively stable ketoamine compound/Amadori compound and heyns product in case of fructose. Since this reaction does not require the participation of enzymes, the variables which regulate it \textit{in vivo} are the degree and duration of hyperglycemia, the half-life of the protein, its reactivity in terms of free amino groups, and cellular permeability to glucose. In \textit{in vivo} conditions, the Amadori...
product reaches equilibrium after approximately 15–20 days and, through irreversible links, accumulates on both short-lived and long-lived proteins (Lapolla et al., 2005).

**Intermediate Stage:**
In the second stage, the Amadori compound further undergoes a series of dehydration and fragmentation reactions generating a variety of carbonyl compounds with some formation of initial cross-linked protein species. Carbonyl compounds are generally more reactive than the original carbohydrate and act as propagators by reactions with free amino groups. Among the most active enhancers of the reaction are α-dicarbonyls such as methylglyoxal, glyoxal, glucosones, deoxyglucosones and dehydroascorbate (Thornalley et al., 1999).

**Late Stage:**
In the late stage, these propagators again react with free amino groups and, through oxidation, dehydration and cyclization reactions, form yellow-brown, often fluorescent, insoluble, irreversible compounds, usually called Advanced Glycation End-Products (AGEs), sometimes known as “melanoidins”, which accumulate on long-lived proteins and cause damage and extensive protein cross-linking. AGEs are characterized by a wide structural and physicochemical diversity.

Reducing sugars other than glucose can participate in glycation and do so much faster than glucose, which is the least reactive of all sugars. This may explain why glucose has been selected as the major metabolic sugar during evolution (Bunn and Higgins, 1981). Glucose is the major metabolic sugar present in our body. Even in euglycemic normal individual the level of glucose in plasma is between 65 mg/dl to 100 mg/dl. Therefore even in non-diabetic euglycemic individuals’ plasma and other proteins are regularly interacting with glucose and result in formation of glycated end products such as AGEs. Wolffenbuttel et al., (1996) suggested that modification of hemoglobin by advanced glycosylation end products would be a better index for long term glycemia in diabetic patients. While glycation can be detected in physiologic conditions like aging, the reactions are considerably faster and more intensive in the pathophysiologic conditions like the uncontrolled diabetes mellitus associated with persistently elevated blood glucose concentration (Thornalley, 2003). In addition to the multiple pathologies mediated by the *in-vivo* generated AGEs, various exogenous sources such as diet and smoking may also
add significantly to the damage caused by those generated in the body (Koschinsky et al., 1997).

Recent data showed that, in spite of the fact that sugars are the main precursors of AGE compounds, numerous intermediary metabolites, i.e. alpha-oxoaldehydes, also creatively participate in non-enzymatic glycation reactions. Such intermediary products are generated during glycolysis (methylglyoxal) or along the polyol pathway and can also be formed by autoxidation of carbohydrates (glyoxal). As compared to glycation reactions involving molecules like nucleic acid and lipids, protein glycation has been studied extensively showing numerous structural alterations including exposure of thiols, protein compaction, cross linking, fragmentation and susceptibility to proteolysis (Seidler and Seibel, 2000). Glycation by various sugars of a limited number of amino groups in proteins like hemoglobin, albumin and low density lipoproteins induce number of alterations in proteins and loss of biological activity (Turk, 2001). These include conformational alterations, exposure of hydrophobic residues and thiols, loss in allosteric sensitivity (Bunn and Briehl, 1970), ligand binding (McDonald et al., 1979) and receptor recognition. In diabetes mellitus, protein glycation and glucose auto-oxidation may generate free radicals, which in turn catalyzes lipid peroxidation (Baynes, 1991).

Fig. 1: Glycation of a protein by glucose and subsequent formation of AGEs (Ahmed, 2005).
ADVANCED GLYCATION END PRODUCTS (AGEs):

AGEs are complex, heterogenous molecules that cause protein cross-linking, exhibit browning and generate fluorescence. The formation of AGEs in-vitro and in-vivo is non-enzymatic and dependent on the turnover rate of the chemically modified target, time and sugar concentration. Persistent hyperglycemia induces abnormal changes such as increase of advanced glycation end products (AGEs) formation, increase of polyol pathway flux, and activation of protein kinase C isoforms (Brownlee, 2001; Evans et al., 2002). Protein modification with AGE is irreversible, as there are no enzymes in the body that would be able to hydrolyze AGE compounds. These structures then accumulate during the lifespan of the protein on which they have been formed. For example, lens crystalline proteins (Stevens et al., 1978), insulin (Dolhofer and Wieland, 1979), proteins of erythrocyte membrane (Miller et al., 1980), bovine serum albumin (Arakawa and Timasheff, 1982), human serum albumin (Shaklai et al., 1984), enzymes (Coradello et al., 1982), high and low density lipoproteins (Kirstein et al., 1990), peripheral nerve myelin (Greene, 1983), elastin (Baydanoff et al., 1987) and immunoglobulin G (Newkirk et al., 2003).

CLASSIFICATION OF AGES:

AGEs are usually grouped into fluorescent, non-fluorescent compounds and cross linking AGEs. Some authors also grouped them into toxic and non-toxic AGEs. Toxic AGEs seem to derive from glycolaldehyde or glyceraldehyde and their structures remain to be elucidated (Sato et al., 2006). Many AGEs fluoresce under UV light and are capable of intra and inter-molecular cross-linking, but not all share these properties (Wautier and Schmidt, 2004). On the basis of these two properties AGEs can be classified into three categories (Fig. 2).

1. **Fluorescent AGE crosslinks:**– Protein-protein crosslinks by these structures in-vivo are thought to be responsible for a major share of the deleterious effects of AGEs in diabetes and aging. Along with brown colour, fluorescence is one of the qualitative properties classically used to estimate these AGEs. For example, pentosidine, crossline, pentodilysine, vesperlysine A, B and C. Pentosidine was first isolated and identified from dura mater collagen and has since been identified in many tissues (Sell et al., 1991).
2. **Non-fluorescent AGE crosslinks:** Although their ease of detection makes them useful markers of AGE formation, the fluorescent AGE cross-links are thought to account for only one percent or less of the total cross-linking structures formed under physiological conditions (Dyer *et al.*, 1991). Thus, the major AGEs responsible for protein-protein cross-linking *in-vivo* are non-fluorescent structures that have not yet been conclusively identified. The structure of three common examples of this class are imidazolium dilysines, alkyl formyl glycosyl pyrrole and arginine-lysine imidazole.

3. **Non-crosslinking AGEs:** Besides the cross-linking AGEs, a number of non-crosslinking AGEs have been reported under physiological conditions. They may have deleterious effects as precursors of cross-links or as biological receptor ligands causing a variety of adverse cellular and tissue changes. Pyrraline, carboxymethyllysine and imidazolones are examples of non-crosslinking AGEs.

**AUTOXIDATIVE GLYCATION AND GLYCOXIDATION:**

Oxidation processes are important in the formation of many AGEs (Lapolla *et al.*, 2005). Two routes have been proposed for AGEs formation. The first involves auto-oxidation of free sugar. Monosaccharides, like glucose, exist in equilibrium with their enediol, which can undergo autoxidation in the presence of transition metals to form an enediol radical (Wolff and Dean, 1987). This radical reduces molecular oxygen to generate the superoxide radical (O$_2^-$) and becomes oxidised itself to a dicarbonyl ketoaldehyde that reacts with protein amino groups forming a ketoimine. This is referred to as autoxidative glycation and is outlined in Fig. 3(a). Ketoimines are similar to, although more reactive, than Amadori products and participate in AGE formation.
Fig. 2: Chemical structure of (a) Fluorescent cross-linking AGEs, (b) Non-fluorescent cross-linking AGEs, (c) Non-cross-linking AGEs (Ahmed, 2005).
The second mechanism involves autoxidation of Amadori products to AGEs as shown in Fig. 3(b). Protein-bound products of the Amadori pattern, in the presence of molecular oxygen and transition metals, are oxidized and give origin to highly reactive proteinenediols generating protein-dicarboxyls and superoxide radical. The protein dicarboxyl compounds can participate in AGE formation and referred to as glycoxidation products. Once formed, the superoxide radicals can be converted to highly reactive hydroxyl radical via the fenton reaction. ROS such as O$_2^•^-$, H$_2$O$_2$, and ’OH may contribute to these reactions, which require trace levels of catalytic redox-active transition metal ions. The process also includes oxidative steps and is therefore called glycoxidation (Bousova et al., 2005; Elgawish et al., 1996; Yim et al., 1995)

RECEPTORS FOR AGES (RAGE)
A number of AGE receptors (RAGE) have been identified in macrophages, endothelial and several other types of cells (Skolnik et al., 1991). Phagocytic cells expressing RAGE internalize and digest AGE modified proteins and therefore these receptors are implicated in protein turnover, tissue remodeling and inflammation (Schmidt et al., 2001; Vlassara, 2001). Expression of RAGE is enhanced in certain cells during diabetes and inflammation. Interaction of AGE with their cellular receptors generates intracellular oxidative stress resulting in the activation of the transcription factor NF-kB and subsequent gene expression which is relevant in diabetic complications (Zill et al., 2001). NF-kB modulates gene transcription for endothelin-1, tissue factor and thrombomodulin and generation of pro-inflammatory cytokines such as interleukin-1a, interleukin-6 and tumor necrosis factor-α (Neumann et al., 1999). There is also enhanced expression of adhesion molecules including vascular cell adhesion molecule-1 and intercellular adhesion molecule-1, in addition to other effects such as increased vascular permeability. The intracellular signaling pathways following activation of RAGE by AGEs are outlined in Fig. 4. The importance of oxidative stress, following binding of AGEs with RAGE in endothelial cells was demonstrated by a study which showed that NF-kB and haem-oxygenase messenger RNA, both markers of oxidative stress, become activated. The same study also showed increased oxidative stress in animals after infusion of AGEs (Yan et al., 1994).
Fig. 3: Reaction schemes for glucose autoxidation (a) and glycoxidation (b) (Ahmed, 2005).
Fig. 4: Mechanism of action of AGEs formed intracellularly (Brownlee, 2001).
BIOLOGICAL EFFECTS OF AGE FORMATION:
AGEs are formed in excess during aging, diabetes mellitus and renal failure (Schleicher et al., 1997). AGE modifications influence the structural as well as functional properties of proteins. Due to AGE modifications, several enzymes alter their activity. Methylglyoxal-modified serum albumin exhibits impaired esterase activity compared to unmodified albumin (Ahmed and Thornalley, 2005). Moreover cystein proteases like cathepsins are inhibited by methylglyoxal modification at active site of cysteins (Zeng and Davies, 2005). Glycated and crosslinked proteins exhibit an increased resistance to proteasomal protein degradation, and hence results in markedly enhanced biological half-life (Bulteau et al., 2001). In contrast to intracellular proteins, collagen in the extracellular matrix (ECM) has a relatively long biological half-life and is directly exposed to high levels of glucose outside the cell. Indeed, modified collagen becomes more resistant to degradation by metalloproteinases which cause accumulation of AGE-modified collagens in the ECM which mediate its stiffening leading to heart and vessel dysfunction (Badenhorst et al., 2003). Lens crystallins are also a long-lived target, leading to cataracts. Damage to DNA due to AGE formation may cause birth defects (Ulrich and Cerami, 2001). Thus AGEs appear to damage cells by three mechanisms (Fig. 5):

The first is the modification of intracellular proteins/intracellular glycation including, most importantly, proteins involved in the regulation of gene transcription (Giardino et al., 1994; Shinohara et al., 1998). The second mechanism being, these AGE precursors can diffuse out of the cell and modify adjacent extracellular matrix molecules nearby/cross-link formation (McLellan et al., 1994) with changes signaling between the matrix and the cell causes cellular dysfunction (Charonis et al., 1990). The third mechanism being, these AGE precursors diffuse out of the cell and modify circulating proteins in the blood, such as albumin. These modified circulating proteins can then bind to AGE receptors and activate them, thereby causing the production of inflammatory cytokines and growth factors, which in turn cause vascular pathology (Smedsrod et al., 1997; Vlassara et al., 1995).
Introduction

The major ways in which glycation changes protein functions are by:

a) Inhibition of regulatory molecule binding
b) formation of cross linkage of glycated protein
c) trapping of glycated proteins by extracellular matrix
d) decreasing the susceptibility of protein to proteolysis
e) loss of biological activity of enzymes including malate dehydrogenase,
glucose-6-phosphate dehydrogenase, glutathione reductase, glyceraldehyde-3-
phosphate dehydrogenase, catalase and superoxide dismutase (Heath et al.,
1996).
f) abnormality of nucleic acid function
g) increased immunogenicity in relation to immune complex formation (Turk,
2001).

GLYCATION OF HUMAN SERUM ALBUMIN:

Human serum albumin is a major protein component of the serum. Albumin contains 585
amino acids and has a molecular weight of 66 kD. This globular protein contains 18
tyrosines, six methionines, one tryptophan, 17 disulphide bridges, and only one Free
cysteine, (Cysteine-34) (Sugio et al., 1999). Structurally it consists of 67% of the
secondary structure which comprises 28 α-helical segments. Rest of the secondary
structure consists of 10% β-turns and 23% extended peptide chain. The tertiary structure
of HSA is arranged in heart shape in three homologous domains I, II, III (Fig. 6). It

Fig. 5: Harmful effect of AGES (Lapolla et al., 2005).
contains only one tryptophan located in domain II (Coussons et al., 1997; Weber, 1975).
This highly soluble protein is present in human plasma at normal concentrations between
35 and 50 g/L. Human serum albumin is a member of the multi-gene family of proteins
that include α-fetoprotein and human-group specific component. In normal conditions, its
half-life is about 20 days, and its plasma concentration represents equilibrium not only
between its synthesis in the liver and its catabolism, but also its transcapillary escape. It is
produced in the liver at a rate of nearly 0.7 mg/g of liver tissue per hour (Peters, 1970).
Its production is under the control of insulin and somatotropin (Hutson et al., 1987).
Albumin has several important physiological and pharmacological functions. It transports
metals, fatty acids, cholesterol, bile pigments, and drugs. It is a key element in the
regulation of osmotic pressure and distribution of fluid between different compartments.
Its most striking property is its ability to bind an unusually broad spectrum of ligands
(Brown, 1982). These include inorganic cations, organic anions, various drugs, amino
acids, and perhaps most important, physiologically available hydrophobic molecules like
bilirubin, hemin, and fatty acids. As a result, albumin is considered a multifunctional
plasma transport protein.
Albumin is also responsible for storage and transport of a large number of drugs in the
plasma (Bhattacharya et al., 2000). It is also supposed to have a high affinity to metal
ions such as Cu$^{2+}$ and Zn$^{2+}$ and act as an antioxidant in the vascular compartment due to
its scavenging of reactive oxygen and nitrogen species generated due to basal aerobic
metabolism normally and formed at an increased rate during inflammation (Halliwell and
Gutteridge, 1990). It is present in the serum in a soluble form and is a major contribute to
80% of plasma colloid pressure (Lundsgaard-Hansen, 1986).
It also acts as an important Acid-Base buffer in plasma. HSA non-enzymatically reacts
with glucose to form a stable glycated albumin (Shaklai et al., 1984). This process is
especially elevated in Diabetes (Dolhofer and Wieland, 1980) due to increased glucose
concentration in plasma. This process of non-enzymatic glycation proceeds in a glucose
concentration, incubation period and temperature dependent manner (Baynes et al.,
1984). The principle site of glycation of HSA is lysine-525, but the lysine residues in
positions 199, 281, and 439 are also susceptible to glycation. In addition there are six
more residues that glycate but with much less efficiency (Shaklai et al., 1984).
**Fig. 6:** Schematic drawing of the HSA molecule. Each subdomain is marked with a different colour (yellow, Ia; green, Ib; red, IIA; magenta, IIb; blue, IIIa; and cyan IIIb). N- and C- termini are marked N and C, respectively. Arginine 117, lysine 351 and lysine 475 which may be sites for binding long-chain fatty acids are colored white (Sugio et al., 1999).
The *in-vitro* exposure of protein to glucose results in the non-enzymatic covalent attachment of glucose to lysine side chains in a manner that observed *in-vivo*. HSA is typically three times more glycated than the rest of the population in conditions of hyperglycemia (Bourdon *et al.*, 1999). In diabetes, HSA may rise from 6-10% to 20-30% and hence serves as the indicator of glycation (Nakajou *et al.*, 2003). HSA also represents the major and predominant circulating antioxidant in plasma known to be exposed to continuous oxidative stress (Soriani *et al.*, 1994). Glucose and free radicals were found to impair the antioxidant properties of the serum albumin (Bourdon *et al.*, 1999). Many studies show the presence of elevated levels of oxidized albumin, in patients with diabetes mellitus (Suzuki *et al.*, 1992), aging (Era *et al.*, 1995), patients with chronic hepatitis C (Rigamonti *et al.*, 2003), oxidized albumin is a reliable marker of oxidative stress in hemodialysis patients (Mera *et al.*, 2005) and many other diseases.

**INHIBITION OF GLYCATION:**

Body has several humoral and cellular defence mechanisms to protect tissues from deleterious effects of glycation reaction and AGE accumulation. These include the glyoxalase systems (I and II) that catalyses the deglycation of methylglyoxal to D-lactate (Thornalley, 1998). The discovery of deglycating enzymes has implications for the repair of protein damage by fructose (Monnier, 2005). A variety of plasma amines may react with sugar and Amadori carbonyl groups to reduce AGEs. Numerous compounds have been investigated for anti-glycation activity and the various sites where potential anti-glycation or AGE compounds could act are outlined in Fig. 7. Currently several strategies are employed to control protein glycation.

(i) Block free amino groups on proteins, preventing glycation by free sugars. However, the biological effect of reducing free protein amino groups is not known.

(ii) Block carbonyl groups on reducing sugars, Amadori products and dicarbonyl intermediates (3-deoxyglucosone, methylglyoxal, etc.) effectively reducing glycation and/or AGE formation. Again, the effect of reducing available carbonyl groups *in-vivo* may not be desirable.
(iii) Antibodies may be used to block Amadori products. This approach has the advantage of specificity compared to use of compounds that merely recognize carbonyl groups.

(iv) Chelation of transition metals by ceruloplasmin may reduce glycation-derived free radicals. However, many transition metals have important physiological functions and their complete removal may have undesirable consequences.

(v) Antioxidants may protect against free radicals derived via autoxidative glycation, glycoxidation and AGEs.

(vi) Enzymes (Amadoriases) may be used to deglycate Amadori products or inactivate intermediates such as 3-deoxyglucosone.

(vii) AGE-cross-link breakers offer the potential of reversing diabetic complications although their precise mechanism of action is still unclear.

(viii) RAGE blockers could prevent interaction of AGEs with RAGE to suppress the cellular and inflammatory changes associated with the development of diabetic complications.

**DIABETES:**

Diabetes mellitus (DM) is a metabolic disorder characterized by chronic hyperglycemia with disturbances of fat, carbohydrate and protein metabolism resulting from defects in insulin secretion and/or insulin action (Turko *et al.*, 2001). The recent statistics indicate that the global prevalence of DM, estimated as 366 million in 2011, will increase up to 522 million by 2030 (Whiting *et al.*, 2011). Diabetes is the fifth leading cause of death in the US and the number of people with diabetes in the world is expected to approximately double between 2000 and 2030 (Wild *et al.*, 2004). India has world's largest number of diabetic subjects and the prevalence of diabetes and impaired glucose tolerance were 12.1% and 14.0% respectively, with no gender difference (Ramachandran *et al.*, 2001).
Fig. 7: Potential sites where pharmacological compounds may act to inhibit protein glycation and AGE-mediated damage (Ahmed, 2005).
The prevalence of diabetes in India is about three times higher in urban population compared to rural population and also the prevalence of diabetes varies widely across the nation, a very high prevalence (16.3%) was reported in Thiruvananthapuram in Kerala State in the year 1999, in the same year, a prevalence of 8.3 per cent was reported from Guwahati (Moebus et al., 2010; Ramachandran et al., 2001). Depending on the etiology of DM, factors contributing to hyperglycemia may include reduced insulin secretion, decreased glucose utilization and increased glucose production (Kasper et al., 2005). Distinct types of diabetes mellitus (DM) are caused by complex interactions of genetics, environmental factors and lifestyle choices. In the United States, DM is the leading cause of end-stage renal disease, non-traumatic lower extremity amputations and adult blindness.

**CLASSIFICATION OF DIABETES:**

Classification of DM is on the basis of pathogenic process that leads to hyperglycemia.

- Type 1 diabetes results from β-cell destruction, usually leading to absolute insulin deficiency.
- Type 2 diabetes results from a progressive insulin secretory defect on the background of insulin resistance, impaired insulin secretion and increased glucose production.
- Other specific types of diabetes due to other causes, e.g. genetic defects in β-cell function, genetic defects in insulin action, diabetes of the exocrine pancreas (such as cystic fibrosis), endocrinopathies (e.g. acromegaly, hyperthyroidism) and drug or chemical induced (such as nicotinic acid, protease inhibitors) or due to infections (e.g. congenital rubella).
- Gestational diabetes mellitus include impaired glucose tolerance during pregnancy.

**DIABETIC COMPLICATIONS:**

The complications of DM affect many organ systems and are responsible for the majority of morbidity and mortality related to the disease. One of the most prevalent metabolic syndromes world-wide, diabetes mellitus (DM), is characterized by hyperglycemia resulting in short-term metabolic changes in lipid and protein metabolism and long-term irreversible vascular and connective-tissue changes. These changes include diabetes-specific complications such as retinopathy, nephropathy, neuropathy and complications
of the macro-vasculature such as atherosclerosis; potentially resulting in heart disease, stroke and peripheral vascular disease (Hudson, 2002).

In diabetic subjects, hyperglycemia is widely recognized as the major cause of diabetic secondary complications due to over generation of ROS (Fig. 8) (Palm et al., 2003). Several hypotheses relating to hyperglycemia have been proposed. Four main hypotheses as shown in Fig. 9 are: (i) Increased polyol pathway flux, (ii) Increased advanced glycation end product (AGE) formation, (iii) Activation of protein kinase C isoforms, and (iv) Increased hexosamine pathway flux.

1. Diabetic retinopathy:
Diabetic retinopathy is one of the most important microvascular complications in diabetes and is a leading cause of acquired blindness among the people of occupational age (L’Esperance et al., 1990). In a large population-based study, prevalence of any degree or proliferative retinopathy was highest in the younger-onset, insulin-taking diabetic patients and lowest in older-onset group not taking insulin (Klein et al., 1984). The prevalence of diabetic retinopathy increases with duration of diabetes. Diabetic retinopathy involves both morphological and functional changes in the retinal capillaries, including basement membrane thickening, loss of pericytes, increased permeability and vascular dysfunction. AGEs have been detected in retinal blood vessel walls and contribute towards vascular occlusion and increased permeability of retinal endothelial cells causing vascular leakage (Beisswenger et al., 1995). AGEs exert their effect on microvascular endothelial cells and pericytes by upregulating levels of their RAGE messenger RNA (Tanaka et al., 2000). AGEs may cause loss of pericytes and death of endothelial cells in diabetic retinopathy. The role of AGE in the development of diabetic retinopathy and the effect of the AGE-formation inhibitor, aminoguanidine, has been examined in animal models (Hammes et al., 1995).
**Fig. 8:** Diabetic secondary complications due to hyperglycemia (Brownlee, 2001).

**Fig. 9:** Potential mechanism by which hyperglycaemia-induced mitochondrial superoxide overproduction activates four pathways of hyperglycaemic damage (Brownlee, 2001).
2. Diabetic nephropathy:

Diabetic nephropathy is a leading cause of end stage renal disease, and accounts for disabilities and the high mortality rate in patients with diabetes (Krolewski et al., 1991). Development of diabetic nephropathy is characterized by glomerular hyperfiltration and thickening of glomerular basement membranes, followed by an expansion of extracellular matrix in mesangial areas and increased urinary albumin excretion rate. Diabetic nephropathy ultimately progresses to glomerular sclerosis associated with renal dysfunction (Sharma and Ziyadeh, 1995).

Serum AGE levels reflect the severity of diabetic nephropathy and their measurement can predict the histopathological conditions (Berg et al., 1997). Circulating serum AGE level is so markedly increased in patients with diabetic nephropathy and renal insufficiency that it cannot be cleared by the kidneys (Turk, 2001). A number of studies have demonstrated that aminoguanidine decreased AGE accumulation and plasmaprotein trapping in the glomerular basement membrane (Raj et al., 2000).

3. Diabetic neuropathy:

Diabetic neuropathies are a family of nerve disorders caused by diabetes. People with diabetes can, over time, develop nerve damage throughout the body. Diabetic neuropathy is associated with risk factors for other vascular complications such as poor metabolic control, dyslipidemia, hypertension, body mass index, smoking, microalbuminuria and retinopathy (Cameron et al., 2001; Forrest et al., 1997; Tesfaye et al., 1996). Both vascular and metabolic factors have been involved in the pathogenesis of diabetic neuropathy. Studies in human and animal models have shown reduced nerve perfusion and endoneurial hypoxia, which might play a role in nerve dysfunction (Ibrahim et al., 1999). An interaction between AGE-myelin and macrophages may initiate or contribute to the segmental demyelination associated with diabetic neuropathy (Vlassara et al., 1984). Aminoguanidine (AG) treatment inhibits an accumulation of fluorescent AGE in diabetic nerves, and partially prevents demyelination and axonal atrophy probably through the correction of endoneurial microcirculation (Sugimoto and Yagihashi, 1997).
4. Diabetic atherosclerosis:
Atherosclerotic arterial disease may be manifested clinically as cardiovascular disease (CVD). CVD is responsible for about 70% of all causes of death in patients with type-2 diabetes (Laakso, 1999). Conventional risk factors, including hyperlipidemia, hypertension, smoking, obesity, lack of exercise, and a positive family history, contribute similarly to macrovascular complications in type-2 diabetic patients and non-diabetic subjects (Laakso, 1999). AGEs formed on the extracellular matrix results in decreased elasticity of vasculatures, and quench nitric oxide, which could mediate defective endothelium-dependent vasodilatation in diabetes (Bucala et al., 1991). AGE modification of low density lipoprotein (LDL) exhibits impaired plasma clearance and contributes significantly to increase LDL \textit{in-vivo}, thus being involved in atherosclerosis (Bucala et al., 1995). Binding of AGEs to RAGE results in generation of intracellular ROS generation and subsequent activation of the redox-sensitive transcription factor NF-kB which promotes the expression of a variety of atherosclerosis-related genes. Taken together, in diabetes, when fueled by hyperglycemia, AGEs and oxidative stress, the AGE-RAGE axis amplifies vascular stress and accelerates atherosclerosis and neointimal expansion (Naka et al., 2004). Blockade of the AGE-RAGE interaction may lead to a successful reduction of CVD in diabetes. Other study shows a correlation between AGE levels and the degree of atheroma in cholesterol-fed rabbits, and that AG has an anti-atherogenic effect in these rabbits by inhibiting AGEs formation (Panagiotopoulos et al., 1998).

PROTEIN OXIDATION IN DIABETES:
Increasing evidence in both experimental and clinical studies suggests that oxidative stress plays a major role in the pathogenesis of both types of diabetes mellitus. Free radicals are formed disproportionately in diabetes by glucose oxidation, non-enzymatic glycation of proteins, and the subsequent oxidative degradation of glycated proteins. Abnormally high levels of free radicals and the simultaneous decline of antioxidant defense mechanisms can lead to damage of cellular organelles and enzymes, increased lipid peroxidation, and development of insulin resistance. These consequences of
oxidative stress can promote the development of complications of diabetes mellitus (Baynes and Thorpe, 1999; Ceriello et al., 2000). Diabetic patients exhibit elevated levels of intracellular iron and copper ions which in the presence of glycated proteins, have been shown to enhance the generation of free radicals in-vitro (Dean et al., 1991). These highly reactive species in turn are able to induce oxidative degradation of protein in-vitro (Pacifici and Davies, 1991). Glycation is a major source of ROS i.e., generated by oxidative pathways of glycation (Rahbar and Figarola, 2003). Several studies support the idea that glycation and oxidation are closely linked processes; glucose autoxidation plays an essential role in non-enzymatic glycation of protein. AGEs are important source of free radicals resulting from non-enzymatic glycation and oxidation of proteins and lipids (Thomas et al., 2005). Free radicals and glycation are central to chronic diseases, degeneration and ageing. Overproduction of free radicals accelerates cell ageing and is counteracted by antioxidants. The analysis of mechanism generating free radicals and of the reaction of AGEs with cellular metabolism opens new avenues for the delaying of the development of chronic diseases like diabetes and neurodegenerative diseases (Giardino et al., 1998).

**ANTIGLYCATING AGENTS:**

The first compound that has been extensively studied in-vitro and in-vivo to be a powerful inhibitor of AGE formation is aminoguanidine (Brownlee et al., 1986). AG prevents the formation of fluorescent AGEs and glucose derived collagen cross-linking. The mechanism of inhibition of AGE formation by AG involves trapping of reactive dicabonyl intermediates such as methylglyoxal, glyoxal and 3-deoxyglucosone (Thornalley, 2003; Thornalley et al., 2000). In addition to chelating or antioxidant activity, AG also acts as true scavenger of carbonyl compounds (Thornalley et al., 2000). Pyradoxamine, a form of vitamin B6, found to inhibit carboxymethyl lysine formation in-vitro but it does not interact directly with Amadori intermediates but interfere with the post amadori oxidative reactions by binding catalytic metal ions (Chetyrkin et al., 2008; Voziyan et al., 2003). Many studies suggested that metal catalyzed oxidation plays a critical role in glucose induced modification in collagen. Transition metals like Cu^{2+} ions can catalyze both glycation and glycoxidation in concentration dependent manner
Carnosine appears to possess antiglycating, antioxidant and free radical scavenging activity. Carnosine inhibits inactivation and crosslinking of enzymes including superoxide dismutase glycation (Ukeda et al., 2002) and oxidation (Stvolinskii et al., 2003). It was found recently the imidazolium group of histadine on carnosine stabilizes the adduct formation at the primary amino group and hence it may play an important role for an anti-crosslinking agent (Hobart et al., 2004). Some anti-inflammatory compounds such as acetylsalicylic acid, ibuprofen indomethacin were also reported to inhibit glycation by preventing the oxidative stress associated with the formation of AGE (Caballero et al., 2000; Shastri et al., 1998; Sobal and Menzel, 2000). Aspirin was also found to inhibit pentosidine formation (Fu et al., 1994; Urios et al., 2007). Some anti-diabetic drugs metformin and progiatanzone were also reported to be powerful AGE inhibitors (Rahbar et al., 2000). Recently, two new classes of aromatic compounds, derivatives of aryl (and heterocyclic) ureido and aryl (and heterocyclic) carboxamide-phenoxy-isobutyric acids and benzoic acids have been reported to be potent inhibitors of glycation and AGE formation (Rahbar and Figarola, 2003). In-vitro studies showed that they could directly interact with several reactive dicarbonyls such as glyoxal and methylglyoxal. They were also found to be potent chelators of Cu\(^{2+}\) and therefore can suppress hydroxyl radical production during sugar autoxidation and glycation reactions (Rahbar and Figarola, 2003).

Recent studies have highlighted the possible benefits of using plant extracts for decreasing glycation over the currently used drugs (Rates, 2001). Flavonoids like quercetin and rutin represent the most common and widely distributed group of plant phenolics and are abundant in foods. They show important antioxidant and AGE inhibitory properties according to their structure (Farrar et al., 2007). Quercetin has been shown to attenuate diabetic nephropathy in streptozotocin-diabetic rats (Anjaneyulu and Chopra, 2004). Bonnefont-Rousselot (2004) stated that improved antioxidant status is one mechanism by which dietary antioxidant treatment contributes to the prevention and reduction of diabetic complications (Jung et al., 2008).

Garcinol, isolated from Garcinia indica fruit rind has been shown to possess antiglycating property in-vitro along with antioxidant and metal chelating properties (Yamaguchi et al., 2000). Mizutani et al., (2000) isolated resveratrol, a natural phytoestrogen found in
grapes which is found to inhibit AGE induced proliferation and collagen synthesis in vascular smooth muscle. Cyperus rotundus suppresses AGE formation and protein oxidation in a model of fructose-mediated protein glycoxidation (Ardestani and Yazdanparast, 2007). Regarding the significance of glycoxidative stress to diabetic pathology, a supplement of antioxidants to inhibit the process of protein modification appears to be a good strategy for preventing diabetic complications (Rahbar and Figarola, 2003).

**FREE RADICAL BIOCHEMISTRY:**
Free radicals are the chemical species having unpaired electrons that are generated *in-vitro* as well as *in-vivo*. They are highly reactive entities and remain so until and unless their valence shell electrons get paired and attain stability. They are formed from parent molecules via the breakage of a chemical bond keeping one electron by each of its fragment or by cleavage of a radical to generate another radical. Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism. Besides, they are also produced in redox reactions (Halliwell and Gutteridge, 2007). Those reactive molecules containing oxygen are termed as reactive oxygen species (ROS). ROS possess modulatory roles on the physiology of body cells and also act as second messengers causing signal transduction (Myatt, 2010; Tavakoli and Asmis, 2012). It encompasses a diverse variety of chemical species including superoxide anions (O$_2^-$), hydroxyl radicals (·OH), singlet oxygen, hydrogen peroxide (H$_2$O$_2$), alkoxy (RO·), peroxyl (ROO·) and hypochlorous acid (HOCl) (Jakus, 2000).

A great deal of literature indicates that free radicals especially active oxygen-centered ones are highly heterogeneous and highly reactive that can invade on the important biomolecules like proteins, lipids and DNA. This consequently can lead to enzyme inactivation, protein modification (deactivation or hyperactivation), lipid peroxidation, membrane degradation, DNA- strand breakage and base modification and so on. Hence, biological free radicals are potentially reactive enough to damage the neighboring biomolecules and can be causative agents for various diseases, aging and cancer (Baek *et al.*, 2003; Hassan *et al.*, 2012; Valko *et al.*, 2004). These radicals are produced by either *endogenous* sources or by *exogenous* sources (Fig. 10). Endogenously, these radicals are
generated from physiological or bodily actions such as immune response, inflammation, toxicity, infection, excessive exercise, ischemia, cancer and aging etc. In analogous to phosphorylation modification of proteins, redox signaling is emerging in reference to events of oxidation of proteins and hence modification by ROS. Indeed, there are many sources of ROS in the cell namely, nicotinamide adenine dinucleotide phosphate oxidase (Block and Gorin, 2012), xanthine oxidase, uncoupling of nitric oxide synthase, cytochrome P_450 (Cubero and Nieto, 2012; Izyumov et al., 2010). However, one of the main sources of ROS is the mitochondrion within the cell where the \( \text{O}_2^- \) is produced as a byproduct of normal oxidative phosphorylation. Exogenous sources include pollution (air and water), heavy metals (lead, mercury, cadmium etc.), certain drugs (gentamycin, cyclosporine), smoking and radiations etc. These agents after getting into the body via different routes are decomposed or metabolized and trigger generation of various free radicals (Brodin and Roed, 1984). Three major forms of ROS shall now be individually discussed in short.

**Superoxide (O_2^-):** Superoxide anion is created from molecular oxygen by the addition of an electron. Its production mainly occurs inside the mitochondrion during the electron transport chain, when a small number of electrons escape from electron transport chain complexes I and III (Valko et al., 2007). \( \text{O}_2^- \) is responsible for the dismutation and release of \( \text{H}_2\text{O}_2 \), which acts as precursors for 'OH ion formation by the catalysis of metal atoms (Holley et al., 2010). It lacks the ability to penetrate lipid membranes and is therefore enclosed in the compartment where it was produced. The formation of superoxide takes place spontaneously, especially in the electron-rich aerobic environment with the activity of respiratory chain enzymes like flavoenzymes, lipoxygenase and cyclooxygenase (Coughlan et al., 2009). The superoxide radical is produced enzymatically by the reaction with xanthine oxidase.

\[
\text{Xanthine} + \text{O}_2 + \text{H}_2\text{O} \quad \rightarrow \quad \text{Uric acid} + \text{O}_2^- + \text{H}^+ 
\]

**Hydrogen peroxide (H_2O_2):** \( \text{H}_2\text{O}_2 \) is not a free radical but is nonetheless highly important much because of its ability to penetrate biological membranes and is produced by the dismutation of \( \text{O}_2^- \) or by direct reduction of \( \text{O}_2^- \) with two electrons (Topo et al., 2010).

\[
\text{O}_2^- + 2\text{H}^+ \quad \rightarrow \quad \text{H}_2\text{O}_2 
\]
It acts as an intermediate in the production of more reactive ROS molecules including HOCl by the action of myeloperoxidase, an enzyme present in the phagosomes of neutrophils and most importantly, formation of *OH via oxidation of transition metals. It’s important functional role is in intracellular signaling (Sundaesan *et al.*, 1995) and can be removed by at least three antioxidant enzyme systems, namely catalases, glutathione peroxidases and peroxiredoxins (Mates *et al.*, 1999).

\[
H^+ + Cl^- + H_2O_2 \rightarrow HOCl + H_2O
\]

**Hydroxyl radical (’OH):** Due to its strong reactivity with biomolecules, ’OH is probably capable of doing more damage to biological systems than any other ROS (Betteridge, 2000). They are produced by Fenton reaction which involves metal ions like Fe^{2+} and Cu^{2+} with H_2O_2, often bound in complex with different proteins or other molecules (Liu *et al.*, 2012). Transition metals thus play an important role in the formation of ’OH (Halliwell, 1999). Transition metals may be released from proteins such as ferritin and the [4Fe-4S] centre of different dehydrases by reactions with O_2^-. This mechanism, specific for living cells, has been called the *in-vivo* Haber-weiss reaction (Fridovich, 1997).

\[
H_2O_2 + Cu^{+}/Fe^{2+} \rightarrow ’OH + OH^- + Cu^{2+}/Fe^{3+}
\]

**Nitric oxide (NO):** Nitric oxide represents an odd member of the free radical family as it contains unpaired electrons and it is not reactive with various biocellular molecules (Wu *et al.*, 2011). Contrarily it easily reacts with other free radicals (e.g., peroxyl and alkyl radicals), generating mainly less reactive molecules, thus in fact functioning as a free radical scavenger in order to inhibit cellular oxidation of lipids in the cell membranes (Hogg and Kalyanaraman, 1998). The O_2^- and NO react with each other to give OONO^- (peroxynitrite), which is highly cytotoxic (Beckman and Koppenol, 1996). OONO^- may react directly with diverse biomolecules in one- or two-electron reactions, readily react with carbon dioxide to form highly reactive nitroso peroxocarboxylate (ONOOCO_2^-), or protonated as peroxonitrous acid (ONOOH) undergo homolysis to form ’OH and ’NO_2 or rearrange to nitrate (NO_3^-). Peroxynitrite, directly or via its reaction products, may oxidize low density lipoproteins, release copper ions by destroying ceruloplasmin, and generally attack tyrosine residues in different proteins, as observed in many inflammatory diseases (Halliwell, 1997). NO is synthesized enzymatically from L-arginine by NO synthase (Andrew and Mayer, 1999).
ANTIOXIDANT DEFENSE SYSTEM:
Free radicals exert derogatory effects on our cellular structure and functions. Hence, living systems have been equipped with antioxidant defense system comprising of endogenous antioxidant molecules or cellular reductants [glutathione (GSH), sulphhydryl groups (-SH), thioredoxin] and antioxidant enzymes:

1. **Superoxide dismutase (SOD):** It dismutates $O_2^\cdot$ into $H_2O_2$ and oxygen. Superoxide dismutases (SODs) are metalloenzymes and their role is to protect aerobic cells against $O_2^\cdot$ action. They catalyze the conversion of superoxide molecules to $H_2O_2$ and $O_2$ and therefore form one of the cell’s major defense mechanisms against oxidative stress (McCord and Fridovich, 1969).

$$O_2^\cdot + O_2^\cdot + 2H^+ \rightarrow H_2O_2 + O_2$$

2. **Catalase:** Catalase protects cells against $H_2O_2$ generated inside them. It has an important role in the acquisition of tolerance to oxidative and nitrosative stress in cellular adaptive response.

$$2H_2O_2 \rightarrow 2H_2O + \frac{1}{2} O_2$$

3. **Glutathione peroxidase:** Glutathione peroxidase uses the thiol reducing power of glutathione to reduce oxidized lipids and protein targets of ROS. Glutathione Peroxidase
catalyses hydroperoxide reduction using GSH, thus protecting mammalian cells against oxidative damage.

\[ \text{H}_2\text{O}_2 + 2\text{GSH} \rightarrow \text{GSSG} + 2\text{H}_2\text{O} \]

4. **Glutathione reductase:** Rereduction of the oxidized form of glutathione (GSSG) is then catalysed by glutathione reductase. This system traps and nullifies the endogenously generated radicals in various metabolic reactions in virtually all the aerobic living systems.

\[ \text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow 2 \text{GSH} + \text{NADP}^+ \]

**ANTIOXIDANTS:**
Antioxidants are substances that are able to prevent or retard oxidation of lipid, proteins and DNA, and to protect the compounds or tissues from damage caused by oxygen or free radicals. Antioxidants are key line of defense capable of trapping free radicals by preventing radical formation, intercepting radicals from further damage to the body (Cotgreave *et al.*, 1988). Antioxidants also protect against glycation-derived free radicals and may have therapeutic potentials.

Apart from endogenous antioxidants a vast number of dietary agents also act as antioxidants. Currently available synthetic antioxidants like butylated hydroxyanisole, butylated hydroxytoluene, tertiary butyl-hydroquinone, propylgallate and gallic acid esters are known to ameliorate oxidative damages but they are suspected to prompt negative health effects. Hence, strong restrictions have been placed on their application and there is a trend to substitute them with naturally occurring antioxidants (Amarowicz *et al.*, 2000; Ghafar *et al.*, 2010). Research on bioactive principles of essential oils extracted from various herbs and spices has become increasingly popular because essential oils have been discovered to have many functional properties such as antimicrobial, antioxidant and anticancer activities (Leal *et al.*, 2003; Lee and Shibamoto, 2002; Vardar-Unlu *et al.*, 2003). Plants, including herbs and spices, have many phytochemicals which are potential sources of natural antioxidants, e.g. phenolic diterpenes, flavonoids, tannins and phenolic acids (Dawidowicz *et al.*, 2006). Green tea is considered a rich source of phenolic compounds and its consumption is considered to be a factor in the lower incidence of coronary heart disease in the Chinese population.
(Zhang et al., 2008). Similarly, parsley oil and two of its inherent bioactive phenolic compounds (i.e., myristicin and apiol) possess antioxidant activity (Zhang et al., 2006). Therefore, polyphenolic compounds’ health promoting effects reduce the risk of various diseases (Manach et al., 2004) and inhibition of growth of pathogenic bacteria (Giroux et al., 2001) which are often associated with the termination of free radical propagation in biological systems. Thus antioxidant capacity is widely used as a parameter to characterize medicinal plants and their bioactive components. There is growing interest in natural products with combined anti-glycation and antioxidant properties as they may have reduced toxicity.

**PHYTOCHEMICALS:**

Aromatic herbs and spices have been used for a long time in alternative medicine, not only to improve or modify the flavor of foods, but also to avoid its deterioration. **Thymoquinone (TQ)** (2-isopropyl-5-methyl-1,4-benzoquinone), is the main bioactive component of the volatile oil of *N. sativa* (Fig. 11). It has been used as antioxidant, anti-inflammatory and antineoplastic medicines for more than 2000 years (Hosseinzadeh and Parvardeh, 2004; Trang et al., 1993). Generally *Nigella sativa* seeds contain more than 30% fixed oil and 0.40% to 0.45% volatile oil. TQ represents 18.4 to 24% of the *N. sativa* volatile oil (Arslan et al., 2005). TQ can also be found in other plants such as Callitris quadrivalvis, Monarda fistulosa, Juniperus cedrus, Tetraclinis articulata, and Nepeta leucophylla. Quinones are ubiquitous in nature and constitute an important class of naturally occurring compounds found in plants, fungi, and bacteria. Current human exposure to quinones occurs via the diet as well as clinically. Benzoquinones are potentially derivable by oxidation of suitable phenolic compounds. Many of these benzoquinones have important biochemical functions in electron transport systems for respiration or photosynthesis (Dewick, 2001). The pharmacological properties attributed to naturally occurring quinones are thus well established. For example, thymoquinone presents anticonvulsant activity in the petit mal epilepsy (Hosseinzadeh and Parvardeh, 2004). *N. sativa* has been traditionally used as a natural remedy for a number of illnesses and conditions such as diabetes, inflammation, bronchitis, fever, and influenza (Ali and Blunden, 2003). The antioxidant effect of TQ is associated with its potential to alter
“redox state” and its scavenging activity against free radicals through modulation of hepatic and extra-hepatic antioxidant enzymes (Karaman et al., 2010). TQ protects the kidney against ifosfamide, mercuric chloride, cisplatin and doxorubicin-induced damage by preventing renal GSH depletion and anti-lipid peroxidation product accumulation, thereby improving renal functioning (Badary, 1999; Badary et al., 1997; Fouda et al., 2008). Khattab and Nagi (2007) assessed the protective effects of TQ after chronic inhibition of nitric oxide synthesis with N-(omega)-nitro-1-arginine methyl esters and found that treatment with TQ increased GSH to normal levels and inhibited the in-vitro production of superoxide radicals.

**Thymol (TL)** (2-isopropyl-5-methylphenol) is a natural monoterpene phenol derivative of cymene, isomeric with carvacrol, found in oil of thyme (Fig. 12) and has been commonly used in foods mainly for flavor, aroma and preservation and also in folk medicine since the ancient Greeks, Egyptians and Romans (Baser, 1993; Baser, 1994). TL can be used for the treatment of oral infectious diseases because of their inhibitory activity on oral bacteria (Didry et al., 1994; Kohlert et al., 2002). Other plants that contain thymol are Origanum compactum and Thymus glandulosus (Tai et al., 2002).

**Eugenol (EU)** (4-allyl-2-methoxyphenol) is a methoxyphenol compound having a short hydrocarbon chain in its structure (Gulcin, 2011). It is found in virtually all spices but bay leaves and cloves are considered the best sources of it (Fig. 13) (Tai et al., 2002). It is one of the major components constituting about 80-95% of clove oil (Szabadics and Erdelyi, 2000). Pharmacologic studies have demonstrated that EU has anticonvulsant (Dallmeier and Carlini, 1981), local anesthetic (Brodin and Roed, 1984), antistress (Sen et al., 1992), bacteriostatic and bactericidal (Walsh et al., 2003) and antifungal properties (Lee et al., 2007). EU and its isoform, isoeugenol have been documented to be potential inhibitors of copper-dependent oxidation of LDL (Ito et al., 2005). Besides, treatment of EU with fast decaying fruits like strawberries increased their average shelf life and also preserved their nutrient values of sugar and organic acids. The treatment also increased the content of total phenolics, anthocyanins and flavonoids (Wang et al., 2007).
**Fig. 11:** (A) *Nigella sativa* seeds and flower, (B) Chemical structure of Thymoquinone (component of *N. sativa*).

**Fig. 12:** (A) *Thymus vulgaris* plant, (B) Chemical structure of thymol (component of *Thymus vulgaris*).

**Fig. 13:** (A) *Eugenia caryophyllata* plant, (B) Chemical structure of Eugenol (component of *Eugenia caryophyllata*).
OBJECTIVE OF THE PRESENT STUDY:

Glycation is the sequence of non-enzymatic reactions involving interaction between reducing sugars and the nucleophilic groups of proteins and other biomolecules. It is ubiquitous in nature and occurs in the cells of all living organisms, albeit at a very slow rate. The rate of glycation however increases remarkably during hyperglycemia, in diabetes and related disorders. Persistent hyperglycemia induces abnormal changes such as increase of AGEs formation, increase of polyol pathway flux, and activation of protein kinase C isoforms. Glycation is also accompanied by the formation of highly reactive and damaging ROS. Free radicals and glycation end products are known to cause severe protein damage resulting in major structural alterations and loss of biological function.

A large body of evidences indicate that glycation is a key molecular basis of diabetic complications. Hyperglycemia is regarded as the primary cause of diabetic microvascular complications that eventually contribute to diabetic macrovascular disease. Diabetic complications usually arise as a result of non-enzymatic protein glycation which leads to the formation of heterogenous, toxic, and antigenic AGEs. Three routes have been proposed for AGEs formation: (i) autoxidative pathway (sugar gives reactive products by autoxidation), (ii) conventional Amadori rearrangement, and (iii) Schiff base formation. Reactive oxygen species (ROS) such as $O_2^{-}$, $H_2O_2$, and $\cdot OH$ contribute to these reactions, which require trace levels of catalytic redox-active transition metal ions. The process includes also oxidative steps and is therefore called glycoxidation. Regarding the significance of glycoxidative stress to diabetic pathology, phytochemicals that interfere with glycation reactions may be beneficial in restricting the complications accompanying diabetes and related disorders. Restriction/prevention of glycation and oxidative stress are therefore mooted as an effective strategy for alleviation of complications associated with hyperglycemia. These days, scientific interests in the functional components of food, with health protecting potencies, are increasing mainly due to their various biological activities and low cytotoxic effects.

Since glycation- and oxidation-induced biochemical changes are known to participate in the overall diabetic complications, the present study was planned to determine antioxidant activity of TQ, TL and EU and their protective effect against AAPH-induced RBC hemolysis. The study was also extended to determine the possibility of preventing
the glucose-induced modification in HSA, as it being the most abundant protein in human blood, by these phytochemicals in concentration and time dependent manner. Furthermore, increase in glycoxidation products in plasma and tissue proteins suggest that oxidative stress increases in diabetes. Hence, the present study was further extended *ex vivo* to evaluate the serum level of lipid peroxides, protein carbonyls, total antioxidant capacity and electrophoretic pattern of albumin in healthy and diabetic patients. The protective effect of TQ, TL and EU on these parameters was also determined. For this, diabetic serum samples were incubated in absence and presence of TQ-2 (30 μM), TL-2 (30 μM) or EU-2 (0.6 μM) for 21 days at 37°C.
Materials and Methods
Materials

Chemicals

Human serum albumin, thymoquinone, thymol, eugenol, aminoguanidine hydrochloride, diethylene triamine penta-acetic acid (DTPA), bovine serum albumin, sodium azide, superoxide dismutase, cytochrome c, dialysis tubing, agarose, 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,2’-azobis-(2-methylpropionamide) dihydrochloride (AAPH) and 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) were purchased from Sigma Chemical Company, USA. D-glucose, 2,4-dinitrophenyl hydrazine (DNPH), ethanol, sodium carbonate and formaldehyde were purchased from Merck, India. Acrylamide, bis-acrylamide, ammonium persulphate and N,N,N’,N’-tetramethyl ethylene diamine (TEMED) were from Bio-Rad Laboratories, USA. Nitroblue tetrazolium (NBT), 5,5-dithio-bis-2-nitrobenzoic acid (DTNB), sulphanilic acid, 2,4,6-trinitrobenzene sulphonic acid (TNBS), Folin-Ciocalteau reagent, bromophenol blue, gallic acid, ascorbic acid (AA) and sodium dodecyl sulphate were from Sisco Research Laboratories, India. Polystyrene microtitre flat bottom ELISA plates and modules were purchased from NUNC, Denmark. Nitrocellulose syringe filters were purchased from AXIVA Sichem Biotech, India. Silver nitrate, trichloroacetic acid, methanol, sodium chloride, sodium acetate, isopropanol, glacial acetic acid, glycine, EDTA, ethylacetate, potassium ferricyanide, ferric chloride, copper sulphate, sodium potassium tartarate and sodium hydroxide pellets were obtained from Qualigens Fine Chemicals, India. All other chemicals and reagents were of the highest analytical grade available.

Equipments

Spectrophotometer (model, U-2910; Hitachi, Japan), Spectrofluorometer (model, RF-5301; Shimadzu, Japan), Spectropolarimeter (model, JASCO J-815; USA), Centrifuge (model, 3K30; Sigma, Germany), Polyacrylamide gel electrophoresis assembly (Genei, India), Densitometer (model, GS-800; Bio-Rad, USA), ELISA reader (Labsystem Multiskan, Finland), pH meter (model, L1-120; ELICO, India), Gel-doc (Bio-Rad Laboratories, USA) and FTIR spectrophotometer (model, 8300; Shimadzu, Japan) were the major equipments used in this study.
Materials

Collection of blood samples

The protocol used for the study was in accordance with guidelines of institutional ethical committee. Forty healthy and forty type 2 diabetic patients diagnosed using World Health Organization criteria were included in the study. Fasting venous blood for estimation of MDA, protein carbonyl, FRAP and glutathione content were drawn after obtaining verbal consent from type 2 diabetic patients attending outpatient department (OPD) clinics at Rajiv Gandhi Centre for Diabetes and Endocrinology, Jawaharlal Nehru Medical College Hospital, Aligarh Muslim University, India. It was ensured that none of the diabetic patients were suffering from other autoimmune diseases. Normal human blood was obtained from age- and sex- matched healthy individuals (Table 1). All blood samples were collected in plain vials and left for clot formation. Serum was separated and stored in small aliquots at -20°C.

Table 1: Clinical features and demographic profile of healthy and diabetic subjects.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Healthy controls (Mean ± SD)</th>
<th>Type 2 diabetes (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size (n)</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Age (years)</td>
<td>49.9 ± 5.71</td>
<td>51.6 ± 6.91</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>18/22</td>
<td>21/39</td>
</tr>
<tr>
<td>BMI (Kg/m²)</td>
<td>22.3 ± 1.39</td>
<td>28.7 ± 1.6</td>
</tr>
<tr>
<td>Fasting glucose (mg/dl)</td>
<td>89 ± 3.14</td>
<td>167 ± 23.38</td>
</tr>
<tr>
<td>Post prandial glucose (mg/dl)</td>
<td>126 ± 12.34</td>
<td>248 ± 23.36</td>
</tr>
<tr>
<td>HbA₁c</td>
<td>4.8 ± 0.43</td>
<td>7.46 ± 0.54</td>
</tr>
</tbody>
</table>
1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging Activity

The free radical scavenging activity of TQ, TL, EU and standard reference compound i.e., Gallic acid (GA) was analyzed by the DPPH assay as described by Sanchez-Moreno et al., (1998) with minor modification. In this assay, 1 ml of varying concentrations of TQ (0.25-2.0 mg/ml), TL (0.25-2.0 mg/ml) and EU (1-60 µg/ml) dissolved in 1 ml of ethanol, were mixed with 1 ml of ethanol solution of DPPH (0.2 mM). The mixture was vortexed and incubated for 30 min. The optical density of the solution was measured at 517 nm using Hitachi, U-2910 standard. The DPPH radical scavenging activity was calculated from the absorption value by the following equation:

Radical scavenging activity (%) = \[\frac{A\_{\text{DPPH}} - A\_{\text{TEST}}}{A\_{\text{DPPH}}} \times 100\]

Where \(A\_{\text{DPPH}}\) is the absorbance of DPPH without test compound (TQ, TL, EU and GA), and \(A\_{\text{TEST}}\) is the absorbance of DPPH in the presence of test compound.

Reducing Power

Total reducing power was determined as described by Zhu et al., (2004) with some modifications. TQ (0.5-2.0 mg/ml), TL (0.5-2.0 mg/ml) and EU (2.5-10 µg/ml) in 1 ml of ethanol were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide \([K_3Fe(CN)_6]\); the mixture was then incubated at 50°C for 30 minute. 2.5 ml of trichloroacetic acid (10%) was then added to the mixture, which was then centrifuged at 3000 rpm for 10 min. 2.5 ml of supernatant was mixed with 2.5 ml of distilled water and 0.5 ml FeCl₃ (0.1%), and the absorbance was measured at 700 nm. Increase in absorbance indicates increased reducing power of the phytochemical.

Ferric Reducing Antioxidant Power (FRAP) assay

FRAP assay is considered as a very reliable method to assess the total reducing potential of any biological active compound or extract. In the present study, it was carried out by the method of Benzie and Strain (1996) with slight modifications. The method is based on the reduction of a ferric 2,4,6-tripyridyl-s-triazine complex (Fe³⁺-TPTZ) to its ferrous
(Fe$^{2+}$-TPTZ), intensive blue colored form in the presence of antioxidant. 300 mM acetate buffer, pH 3.6, 10 mM TPTZ and 20 mM FeCl$_3$ were mixed in a ratio of 10:1:1 to be a working FRAP reagent. 100 µl of TQ, TL and EU were mixed with 3 ml of FRAP reagent and incubated at 37°C for 30 min. The absorbance at 593 nm was monitored. All reagents were freshly prepared before use.

Ferric reducing antioxidant power of healthy, diabetic and phytochemical treated diabetic serum samples was also determined by the same above protocol. Diabetic serum samples were incubated with three concentrations of TQ, TL and EU for 1 hour at 37°C. The concentrations of TQ and TL were 3 µM, 30 µM and 300 µM while those for EU were 0.06 µM, 0.6 µM and 6.0 µM. 20 µl of each phytochemical was incubated in 80 µl of serum of diabetic patients. 80 µl of healthy and diabetic serum samples incubated with 20 µl of 20 mM phosphate buffer (pH 7.4) served as control. 3 ml of FRAP reagent was added to each tube and incubated at 37°C for 30 min. The absorbance at 593 nm was monitored. Aqueous solution of known Fe (II) concentration was used for calibration (in a range of 100-1000 µmol/l) (Table 2, Fig. 14).

**Blank:** FRAP reagent.

**Table 2** Standards: 1mM standard solution of ferrous sulphate was prepared by mixing 0.278 g of FeSO$_4$.7H$_2$O in 1 litre distilled water. They were diluted as follows to make a series of standards of different molarities.

<table>
<thead>
<tr>
<th>Standard concentration (mM)</th>
<th>FeSO$_4$.7H$_2$O solution (ml)</th>
<th>Distilled water (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>0.2</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>0.4</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>0.6</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>0.8</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>1.0</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

Freeze at -20°C in 0.2 ml aliquots in ependorfs.
Fig. 14: Standard curve for ferrous sulphate for the determination of FRAP equivalent.

**Metal Chelating Activity**

The chelation of ferrous ions by TQ, TL and EU was estimated by the method of Dinis et al., 1994 with slight modifications. Different concentrations of the TQ (12.5-200 µg/ml), TL (12.5-200 µg/ml), EU (3.125-25 µg/ml) and AA (3.125-25 µg/ml) were added to a solution of 1 mM FeCl₂ (0.05 ml). The reaction was initiated by the addition of 1 mM ferrozine (0.1 ml) and the mixture was finally quantified to 1 ml with methanol, shaken vigorously, and left standing at room temperature for 10 min. After the mixture had reached equilibrium, the absorbance of the solution was measured spectrophotometrically at 562 nm.

**AAPH-induced RBC hemolysis assay**

Blood was obtained from healthy human donor and collected into heparinized tubes through the Blood Bank, J. N. Medical College, Aligarh Muslim University, Aligarh. Erythrocytes were separated from plasma and the buffy coat, and washed three times with 5 volumes of phosphate buffered saline (PBS), pH 7.4. During every wash, RBCs were centrifuged at 4000 rpm for 10 min to obtain packed cell preparation (Miki et al., 1987). The packed RBC was suspended in four volumes of PBS solution after the last wash. AAPH, a peroxyl radical initiator, was used for RBC hemolysis. Addition of AAPH to the suspension of washed erythrocytes induces the oxidation of membrane lipids and
proteins, resulting in hemolysis. 0.5 ml of the erythrocyte suspension was mixed with 0.5 ml of PBS solution containing varying amounts of TQ, TL and EU and to this 0.5 ml of 200 mM AAPH was added. The reaction mixture was shaken gently while being incubated at 37ºC for 3 hours. After incubation, reaction mixture was diluted with eight volumes of PBS and centrifuged at 4000 rpm for 5 min. The Absorbance (A) of the supernatant was recorded at 540 nm. Percent inhibition was calculated by the following equation:

\[
\% \text{ Inhibition} = \left(\frac{A_{\text{AAPH}} - A_{\text{TEST}}}{A_{\text{AAPH}}}\right) \times 100
\]

Where \(A_{\text{AAPH}}\) is the absorbance of AAPH at 540 nm and \(A_{\text{TEST}}\) is the absorbance of TQ, TL, EU and AA at 540 nm.

**Estimation of malondialdehyde (MDA) level**

Free radicals, by their unstable and transient nature are difficult to measure directly. Their tendency to cause lipid peroxidation has been used as an indirect measure. Hence, estimation of lipid peroxides (markers of lipid peroxidation) was done by measuring MDA (malondialdehyde), which is a stable end byproduct of lipid peroxidation. MDA is one of the most reliable products to assess the extent of lipid peroxidation. Its level was determined by the procedure of Ohkawa et al., (1979) with some modifications. It is based on reaction of Thiobarbituric acid (TBA) with MDA. One molecule of MDA reacts stoichiometrically with two molecules of TBA at pH 3.5. The pink color chromogen can be measured spectrophotometrically at 532 nm with the production of a pink pigment having an absorbance maximum at 532 nm.

**Preparation of TBA reagent**

TBA reagent was prepared by mixing 0.1 ml SDS (8.1% w/v), 0.75 ml acetic acid (20% v/v, pH adjusted to 3.5 with 5N NaOH) and 0.75 ml TBA (0.8% w/v).
Methods

**Procedure**

Three concentrations of thymoquinone, thymol and eugenol were prepared for *in-situ* study. The concentrations of TQ and TL were 3 µM, 30 µM and 300 µM while those for EU were 0.06 µM, 0.6 µM and 6.0 µM. 20 µl of each phytochemical was incubated in 100 µl of serum of diabetic patients for 1 hour at 37°C. 100 µl of healthy and diabetic serum samples incubated with 20 µl of 20 mM phosphate buffer (pH 7.4) served as control. Then 1.6 ml of TBA reagent was added to all incubated tubes. After vortexing, samples were incubated for 1 hour in 95°C and after cooling with tap water centrifuged at 4,000 rpm for 10 min. Supernatant was separated and measured spectrophotometrically at 532 nm. TBARS values were expressed as MDA equivalents. Concentration was calculated using extinction coefficient of the TBA reagent = 1.56 x 10^5 mol⁻¹ cm⁻¹.

**MDA concentration is expressed in terms of nmoles/ml of serum.**

**Determination of Reduced glutathione (GSH) content**

Reduced glutathione (GSH) level was determined by method of Ellman, (1959) with slight modifications. 500 µl of serum was precipitated with 500 µl sulphosalicylic acid (4%). The samples were then centrifuged at 1,200 rpm for 10 min at 4°C. 400 µl of supernatant was taken and 400 µl of DTNB (3 mM) and 2.2 ml of phosphate buffer (0.2 N, pH 8.9) was added. The yellow color developed was read at 412 nm and the amount of reduced glutathione was calculated using the molar extinction coefficient value of 1.36 x 10⁴ cm⁻¹ mol⁻¹.

**MODIFICATION OF HUMAN SERUM ALBUMIN BY GLUCOSE:**

Commercial HSA that gave single band in SDS-PAGE was used without purification. Lyophilized HSA was reconstituted with 20 mM phosphate buffer (pH 7.4) to make a stock solution of 3 mg/ml and stored at -20°C. In order to induce glycation, the protocol used by Miyazawa *et al.*, (1998) was adopted with slight modifications. HSA 1 mg/ml (15.15 µM), was incubated for 7, 14, 21, and 28 days at 37°C in dark with 100 mM glucose in 20 mM phosphate buffer (pH 7.4). The reaction mixtures were pre-filtered.
through a nitrocellulose filter 0.2 µm pore-sizes in pre-autoclaved boxes in order to maintain sterile conditions during incubation. Samples incubated without sugar similarly served as control. After incubation, samples were extensively dialyzed against PBS in order to remove excess of sugars and stored at -20ºC for further analysis. Protein concentration was measured by Lowry et al., (1951).

**IN-VITRO GLYCATION OF HSA WITH GLUCOSE ALONG WITH VARIOUS COMPOUNDS:**

HSA was incubated with 1mM aminoguanidine (standard anti-glycating agent) in the presence of 100 mM glucose. HSA was also incubated with varying concentrations of thymoquinone (3, 30 and 300 µM), thymol (3, 30 and 300 µM) and eugenol (0.06, 0.6 and 6.0 µM) in presence of 100 mM glucose. They are incubated under similar conditions for 4 weeks. HSA incubated without sugar served as control. Before further analysis each sample was extensively dialyzed against the buffer in order to remove excess of sugars.

**UV-visible Spectrophotometry**

The ultraviolet absorption spectra of native, glycated and phytochemical treated glycated HSA samples were recorded in the wavelength range 200–400 nm on a Hitachi U-2910 spectrophotometer, using a cuvette of 1 cm pathlength. One milligram of the sample in a total volume of 3 ml was taken for spectral analysis.

**Fluorescence spectroscopy**

Native, glycated and phytochemical treated samples were analyzed by measuring intrinsic fluorescence at 25ºC on Shimadzu (RF 5301-PC) spectrofluorometer. The fluorescence of tryptophan residue (Tryptophan-214) in all samples was monitored with excitation at 285 nm and emission range was taken at 290 – 400 nm (Shaklai et al., 1984). The concentration of protein samples was taken as 2 µM.

**AGE-specific Fluorescence**

AGEs formation were measured by determining the fluorescence by excitation at 370 nm and emission between 400 - 500 nm (Lapolla, 1992) using Shimadzu (RF 5301-PC).
spectrofluorometer. Loss of fluorescence intensity (FI) was calculated using the following equation:

\[
\% \text{ Loss of FI} = \frac{[\text{FI}_{\text{native HSA}} - \text{FI}_{\text{glycated HSA}}]}{\text{FI}_{\text{native HSA}}} \times 100
\]

**Circular dichroism (CD) studies**

CD spectra were recorded on JASCO spectropolarimeter (J-815) calibrated with D-10-camphorsulfonic acid. The measurements were made at 25°C with a thermostatically controlled cell holder attached to Neslab’s RTE 110 water bath with a temperature accuracy of ± 0.1°C. Far-UV CD was used to measure the changes in the secondary structure of HSA (3.0 µM) in 20 mM phosphate buffer (pH 7.4). Protein samples were placed in cylindrical quartz cuvettes of pathlength 1mm. Each spectrum was the result of average of four scans. CD measurements were performed on HSA samples withdrawn every week from the reaction mixture under incubation for 4 weeks kept at 37°C. The results were expressed as mean residue ellipticity (MRE) in deg cm² dmol⁻¹ which is defined as:

\[
\text{MRE} = \frac{\theta_{\text{observed}} (\text{mdeg})}{10 \times (n) \times C_p \times l}
\]

Where, \(\theta_{\text{observed}}\) is the CD in milli-degree, \(n\) is the number of amino acid residues \((585 - 1 = 584)\), \(l\) is the path length of the cell, and \(C_p\) is the concentration of protein in moles/litre. Helical content was calculated from the MRE values at 222 nm using the following equation (Chen *et al.*, 1972).

\[
\% \alpha\text{-helix} = \frac{[\text{MRE}_{222\text{nm}} - 3030]}{2340} \times 100
\]

**Fourier Transform Infra-red spectroscopy (FTIR)**

To confirm the interaction between HSA, aminoguanidine and phytochemicals (TQ, TL and EU) in absence and presence of glucose, FTIR was conducted. FTIR spectra of native, glycated and phytochemical treated samples were recorded on Shimadzu-8300 FTIR spectrophotometer (Tokyo, Japan) in the spectral range of 400 – 4000 cm⁻¹. Samples to be analyzed were loaded between two potassium bromide discs by hydraulic pressing.
Determination of Protein Concentration

The protein content in all the samples was estimated by the method of Lowry et al., (1951). The protein estimation by this method utilizes alkali (to keep the pH high), Cu$^{+2}$ ions (to chelate proteins) and tartarate (to keep the Cu$^{+2}$ ions in solution at high pH).

Folin-Ciocalteau reagent

The Folin-Ciocalteau reagent was diluted 1:1 with distilled water before use.

Alkaline copper reagent

The components of alkali copper reagent were prepared as follows:

- 2% sodium carbonate in 100 mM sodium hydroxide
- 0.5% copper sulphate in 1.0% sodium potassium tartarate.

The working reagent was prepared fresh before use by mixing components (i) and (ii) in the ratio of 50:1.

Procedure

Aliquots of protein solution were taken in separate tubes and the final volume was made up to 1 ml. To this, 5 ml of alkaline copper reagent was added. This solution was kept as such at room temperature for 10 minutes followed by addition of 1 ml Folin-Ciocalteau reagent. The test tubes were vortexed followed by incubation for 30 minutes at room temperature. The resulting blue color intensity was read at 660 nm against the reagent blank using spectrophotometer. A standard curve was prepared using BSA as standard (Fig. 15).

![Fig. 15: Bovine serum albumin (BSA) standard curve using Lowry’s method of protein estimation.](image)
Methods

Ketoamine estimation by Nitrobluetetrazolium assay
Glycation of HSA or level of Amadori product was determined by an established colorimetric procedure using NBT (Mashiba et al., 1992) with slight modifications. Native, glycated and phytochemical treated HSA samples (50 µl) were added to the wells of 96-well microtitre plate in duplicate. One hundred µl of NBT reagent (250 µM in 0.1 M carbonate buffer, pH 10.35) was added to each well and incubated at 37°C for 2 hours. The plate was read in a microplate reader at 550 nm. The amount of ketoamine in all samples was determined using 12640 cm⁻¹M⁻¹ as molar extinction coefficient of monoformazan.

Determination of Protein bound carbonyl groups
Carbonyl content of native, glycated HSA and phytochemical treated glycated HSA was estimated by a published procedure of Levine et al., (1990) with slight modifications. Briefly, 200 µl aliquot (containing 0.1 mg of protein) was mixed with 400 µl of 7 mM dinitrophenyl hydrazine (DNPH) in 2M HCl. The mixtures were run in duplicate and the control protein samples were devoid of DNPH. After incubation for 1 hour at room temperature, 500µl trichloroacetic acid (4% w/v) was added to precipitate DNP-hydrazones. Mixture was centrifuged for 5 min at 14,000 rpm. The pellet was dispersed in 1 ml ethanol-ethylacetate (1:1 v/v), in order to remove unreacted DNPH and centrifuged. After 3 such washes, the pellet was dissolved in 0.6 ml of 6M guanidinium hydrochloride solution in 20 mM phosphate buffer already adjusted to pH 2.3 with trifluoroacetic acid. The hydrazones were dissolved completely only by overnight freezing at -20ºC and thawing. From the solution, 200 µl aliquot was taken into a microplate and read at 379 nm by a microplate reader. The results were expressed as the number of nmoles of carbonyl per mg of sample protein using a molar absorption coefficient 22,000 M⁻¹cm⁻¹. Samples were analyzed against a blank of 1 ml of 6 M guanidinium hydrochloride solution.

Similar procedure was used for the estimation of protein carbonyl contents in the serum of healthy and diabetic patients in case of ex vivo studies. Three concentrations of thymoquinone, thymol and eugenol were prepared for ex vivo study. The concentrations of TQ and TL were 3 µM, 30 µM and 300 µM while those for EU were 0.06 µM, 0.6 µM.
and 6.0 µM. 100 µl of diabetic serum was incubated with 20 µl of each phytochemical for 1 hour at 37ºC. 100 µl of healthy and diabetic serum samples incubated with 20 µl of 20 mM phosphate buffer (pH 7.4) served as control. After vortexing, all serum samples followed the Levine et al., (1990) protocol.

**Determination of Free Amino Groups**

The free amino groups of native, glycated and compound treated glycated HSA were measured using TNBS procedure described by Haynes et al., (1967). To 0.2 ml of glycated protein, 1.8 ml of sodium tetraborate buffer pH 9.3 was added. Further 20 µl of 0.13 M TNBS solution was added. The reaction mixture thus formed was incubated at 37ºC for 30 min. The absorbance of the solution was read at 420 nm against a blank.

**Detection of superoxide anion radicals in glycated HSA in presence of phytochemicals**

Superoxide radical (O•−) was measured by the cytochrome c reduction method (Beauchamp and Fridovich, 1971). The effect of AG (1 mM) and varying concentrations of TQ (3, 30 and 300 µM), TL (3, 30 and 300 µM) and EU (0.06, 0.6 and 6.0 µM) on the generation of superoxide radicals during glycation of HSA was determined. The reaction tube contained HSA (15.15 µM), glucose (100 mM), DTPA (0.01 mM), cytochrome c (200 µM) in absence and presence of AG, TQ, TL or EU. Reaction was carried out at 37ºC for 20 min in 100 mM phosphate buffer (pH 7.4). The reduction rate was monitored at 550 nm.

**Sodium dodecyl sulphate polyacrylamide gel electrophoresis**

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis was performed as described earlier by Laemmli, (1970) using slab gel electrophoresis apparatus. The following stock solutions were prepared:

*Acrylamide-Bisacrylamide (30:0.8)*

A stock solution was prepared by dissolving 30 g of acrylamide and 0.8 mg of bisacrylamide in distilled water to a final volume of 100 ml. The solution was filtered and stored at 4ºC in an amber color bottle.
Methods

Resolving Gel Buffer
A stock solution was prepared by dissolving 9.08 g Tris base in 40 ml distilled water. The pH was adjusted to 8.8 and the final volume brought to 50 ml with distilled water.

Electrode buffer
3 g Tris base, 14.4 g glycine and 1.0 g SDS were dissolved in distilled water, pH adjusted to 8.3 and final volume was made to 1 litre.

Procedure for PAGE
Thoroughly cleaned glass plates separated by 1.5 mm thick spacer were sealed with 1% agarose from sides and bottom. The resolving gel mixture (10%) was prepared by mixing the components listed in table 3 and poured between the glass plates and allowed to polymerize at room temperature. Protein samples mixed with one fourth of sample dye (10% SDS, 50% glycerol, 1M Tris, pH 6.8, 5% β-mercaptoethanol and 1% bromophenol blue as a tracking dye) were boiled at 100°C for 5 minutes and then loaded into the wells, and electrophoresis was carried out at 80 volts for 3-4 hours in electrode buffer.

For ex vivo studies, diabetic serum samples were incubated in absence and presence of TQ-2 (30 µM), TL-2 (30 µM) or EU-2 (0.6 µM) for 14 days at 37°C. Effect was also compared with HSA incubated in same conditions in presence of 20 mM glucose to check the effect on glycation reaction in excess glucose and the preventive/inhibitory effect by the phytochemicals.

Table-3: Recipe for 10% resolving gel

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>4.0 ml</td>
</tr>
<tr>
<td>Acrylamide-bisacrylamide (30: 0.8)</td>
<td>3.3 ml</td>
</tr>
<tr>
<td>Resolving gel buffer</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>1.5% ammonium persulphate</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>4.0 μl</td>
</tr>
</tbody>
</table>
Serum of healthy individuals without any treatment served as control. They were analyzed by 10% SDS-PAGE in the presence of thiol-reductant β-mercaptoethanol followed by silver staining.

**Silver Staining**

The procedure described by Merril *et al.* (1982) was followed. After electrophoresis the protein bands were fixed by rapidly immersing in a mixture of 40% methanol and 13.5% formaldehyde for 10 min with instant shaking. The gel was washed with distilled water twice at an interval of 5 min. Then the gel was immersed in 0.02% sodium thiosulphate solution for 2 min. The gel was again rinsed twice with distilled water at an interval of 20 second. This was followed by incubation with 0.1% silver nitrate solution for 10 min. The gel was rinsed with distilled water briefly, immersed in developer solution (3% sodium carbonate solution containing 50 µl formaldehyde and 2 ml of 0.02% sodium thiosulphate) for 15 min or until properly stained. The reaction was stopped by transferring the gel to stopper solution (25% isopropanol solution containing 10% acetic acid glacial). The gel was washed twice with distilled water and finally stored in distilled water.

**Densitometric Analysis**

Image analysis of gels was performed to show the inhibition of cross-linked AGEs and/or fragmentation in different concentrations of TQ, TL and EU. Since protein concentration loaded in each well of gel remains same throughout the whole study i.e. 10 µg, therefore, the area selected for performing densitometry of each band was taken to be same or equal. Bands were compared within the same gel and sufficient background was included.