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 as shown by higher inhibition of RBC hemolysis. Similarly, reducing power and FRAP value was found to be higher in case of EU as compared to TL and TQ. The order of antioxidant activity of these compounds was same in all antioxidant parameters tested viz. TQ<TL<EU. 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 ε-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
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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
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 enediol 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...
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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.