Dietary flavonoids inhibit the glycation of lens proteins: implications in the management of diabetic cataract

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Abstract

The intervention of functional foods as complementary therapeutic approach for the amelioration of diabetes and sugar induced cataractogenesis is more appreciated over the present day chemotherapy agents owing to their nontoxic and increased bioavailability concerns. Dietary flavonoids, a class of bioactive phytochemicals is known to have wide range of biological activities against variety of human ailments. In the present study, we demonstrate anti-cataract effect of eight dietary flavonoids in sugar induced lens organ culture study. We present data on processes like inhibition of glycation-induced lens cloudiness, lens protein aggregation, glycation reaction and advanced glycation end products formation that can act as biochemical markers for this disease. The selected flavonoids were also tested for their aldose reductase (AR) inhibition (experimental and in silico). The molecular dynamics simulation results shed light on mechanistic details of flavonoid induced AR inhibition. The outcome of the present study clearly focuses the significance of kaempferol, taxifolin and quercetin as potential candidates for controlling diabetic cataract.

Keywords Diabetic cataract · Dietary flavonoids · Glycation · Aldose reductase · Molecular docking · Molecular dynamics

Introduction

Global technology driven industrialization accelerated the economic growth at the cost of deteriorating human health leading to a paradigm shift in the life style of human beings. Overindulgent urban lifestyle perhaps invited or aggravated series of human ailments. In the current situation, global increase in the diabetic population is growing very fast,

where over 366 million people are suffering from altered sugar homeostasis and the projections that over 552 million people may be victim of diabetes up to 2030. Therefore, there is an urgent need of novel approaches for effective therapeutic management of diabetes and related complications including cataract. Cataract, which is also called as opacification or cloudiness of lens accounts for 51% of total blindness worldwide. Clinical studies shows that the humans suffering from diabetes are more prone for developing cataracts and thereby adversely affecting the vision (Negre-Salvayre et al. 2009).

Glycation induced lens damage has been recognized as one of the leading factor for development of diabetic cataract. In glycation, the excess levels of sugar in diabetic patient undergo reaction with important biological macromolecules without the aid of an enzyme. Chemically, glycation involves a series of complex cascade of events which involves reaction between reducing sugars and free amino groups of protein or free amine group of lipids or DNA (Singh et al. 2001). In the first step of protein glycation, there is formation of Schiff base between aldehyde or keto group of glucose and free amino group (lysine or arginine) of proteins. In the subsequent phases, several rearrangements take place in the intermediate products leading to formation

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of stable Amadori products (Baynes et al. 1989). Gradually, in due course, the Amadori products undergoes series of complex chemical reactions and rearrangements to form stable, heterogeneous products called as advanced glycation end products (AGEs). Sizable amount of literature describes the role of AGEs in the genesis and progression of both diabetic and age-related cataract (Singh et al. 2001). The AGEs bring modification in tertiary structure of lens proteins and subsequently affects lens fibre membrane integrity, which leads to covalent crosslinking of lens proteins like crystallins that eventually form dense protein aggregates (Shamsi et al. 2000). Therefore, measuring the levels of AGEs in the cataractous lens might serve as a diagnostic marker in the advancement of cataractogenesis.

As stated previously, the AGEs (a product of glycation) involves cross linking of lens proteins resulting in formation of high molecular weight (HMW) protein aggregates. Of note, the formation of HMW protein aggregates is more apparent in diabetic cataract (Abraham 1991). The HMW protein aggregates generated in the lens accounts for light scattering effect, reduction in refractive index, lens opacity, alterations in the dynamic state of lens proteins, and ultimately loss of visual acuity (Moreau and King 2012). Measuring the size of the glycation mediated protein aggregates might provide valuable information in relation to the extent of damage to the lens tissue and can be employed as a prognostic and/or diagnostic marker in aging as well as glycation induced cataractogenesis (Patil et al. 2016a).

Polyol pathway is one among the important pathways of glucose metabolism. Under normal sugar homeostasis around 3% glucose is metabolized via this pathway, however, under hyperglycaemic conditions, over tenfold glucose (30%) is diverted through polyol pathway (Gacche and Dhole 2011a). Aldose reductase (AR; E.C.1.1.1.21) is the first key enzyme of the polyol pathway that reduces excess D-glucose into D-sorbitol (a sugar alcohol) by utilizing NADPH as a coenzyme (Gacche and Dhole 2011a). The excess amount of sorbitol generated in diabetic state accumulates in the lens tissue owing to its slow conversion by sorbitol dehydrogenase. The excess accumulation of sorbitol generates an osmotic pressure which results into osmotic swelling and bursting of the lens tissue leading to formation of cataract (Zhu 2013). Therefore, the inhibition of AR has been recognized as a novel target for design and development of effective anti-diabetic agents, perhaps also helpful for amelioration of diabetic complications like cataract (Gacche and Dhole 2011b; Zhu 2013).

Flavonoids are member of a comprehensive category of polyphenols (secondary metabolites) that have demonstrated wide biological activities with myriad of health effects (Kumar and Pandey 2013). Continuation of our ongoing efforts of exploring the potential of dietary flavonoids as anti-cataract agents (Patil et al. 2016a), here we

have selected a panel of eight dietary multi hydroxylated flavonoids such as fisetin, luteolin, kaempferol, taxifolin, quercetin, myricetin, catechin, and epigallocatechin gallate for assessing their anti-cataract potential using sugar induced goat lens model studies and various biochemical parameters like inhibition of glycation, protein aggregation, AGE fluorescence, lens opacity and experimental as well as in silico aldose reductase inhibition have been carried out so as to ascertain their structure activity relationship.

Materials and methods

A set of eight selected flavonoids like Fisetin (3, 3', 4', 7-tetrahydroxy flavone), Luteolin (3', 4', 5, 7-tetrahydroxy flavone), Kaempferol (3, 4', 5, 7-tetrahydroxy flavone), Taxifolin (3, 3', 4', 5, 7-pentahydroxy flavanone), Quercetin (3, 3', 4', 5, 6-pentahydroxy flavone), Myricetin (3, 3', 4', 5, 5', 7-hexahydroxy flavone), Catechin [(−)-trans-3, 3', 4', 5, 7-pentahydroxy flavane] and Epigallocatechin gallate [(−)-cis-3, 3', 4', 5, 5', 7-hexahydroxy-flavane-3-gallate] were obtained from Sigma-Aldrich Co. St. Louis MO USA, KRB (Kreb Ringer Bicarbonate) buffer, Aminoguanidine hydrochloride, NADPH, 2, 4-dinitrophenyl hydrazine (2, 4-DNPH), streptomycin, penicillin, trichloro acetic acid (TCA), etc., were purchased from the Himedia Laboratories Pvt. Ltd. Mumbai (MS), India. All other chemicals, solvents and reagents used were of AR grade and were purchased from commercial sources.

Bovine lens organ culture study

The protocol of bovine lens organ culture study was approved by Institutional Biosafety Committee (IBSC). Fresh goat eyeballs from 6- to 8-month-old animals were obtained from the local slaughter house at Nanded city (MS). After removal from the animals, the eye balls were immediately transferred into KRB buffer (pH 7.5). The antibiotic Penicillin and Streptomycin were used in media buffer to avoid microbial contamination. The procedure for removal of lenses, and their culture conditions were similar to our previous settings (Patil et al. 2016a). In each experimental set, minimum four lenses (from two animals) were used. The flavonoid treated set comprised of lenses incubated individually in KRB buffer (containing a baseline glucose concentration of 10 mM and osmolality of 255–295 mOsm) with further addition of 30 mM glucose (a supraphysiological concentration required for inducing glycation) and 50 µM individual flavonoids. A set of positive control (lens in KRB buffer without additional 30 mM glucose), negative control (lens in KRB with added 30 mM glucose) and a control of standard anti-glycation compound (aminoguanidine 50 µM) were also run simultaneously for comparative analysis. The

lenses in different sets were incubated in CO₂ incubator (95% air, 5% CO₂) at 37 °C temperature. After every 48 h, the media of treated sets was substituted with fresh KRB buffer added with 30 mM glucose, while plane KRB was used in the media of positive control. After the completion of 15 days of incubation, the lenses were observed for morphological changes, opacity, haziness, structural disruption, intactness, etc. The lenses of various sets were digitized using trinocular zooming microscope (Olympus SZ 61TR). The lenses from various sets were further used for various biochemical analyses like profiling of soluble and insoluble protein fractions, AGEs fluorescence, estimation of carbonyl groups, sizing of protein aggregates, etc.

Determination of soluble and insoluble protein fractions of flavonoid treated lenses

The flavonoid treated lenses from various sets were subjected for quantitative profiling of total proteins and the amount of soluble and insoluble protein fractions. In short, for preparing the 10% lens homogenate, the individual lenses from different sets were homogenized in TNEN buffer (pH 8). The mixtures were centrifuged at 10,000g at 4 °C for 30 min. Supernatant (crude lens soluble proteins) was collected and used for further analysis. Lowry method (Lowry et al. 1951), was used for quantification of total proteins (TP) and total lens soluble proteins (TSP). The amount of TP and TSP present was expressed as mg/gm of lens tissues.

Quantitation of protein carbonyl groups

The amount of protein carbonyl groups (as a marker of glycation) were estimated as per the previously described method (Uchida et al. 1998). In brief, the lens proteins from individual sets were incubated with equal volume of 0.1% of 2, 4-DNPH in 2N HCl at 37 °C up to 1 h. Further the precipitation of proteins was achieved by adding 20% trichloro acetic acid (TCA) and washed three times with ethanol/ethyl acetate (1:1). The protein precipitate obtained was solubilised in Tris–EDTA buffer (pH 7.4) containing 8M Urea. The absorbance of the cocktail was recorded at 365 nm. Amount of protein carbonyl groups were calculated using molar extinction coefficient ($\epsilon_{365\text{ nm}} = 21/\text{mM}/\text{cm}$).

Measurement of AGE fluorescence of glycated lens proteins

Fluorescent AGEs generated in the glycated lens proteins were analysed by measuring the fluorescence intensity of AGEs present in TSP (Patil et al. 2016a). Initially, TSP of lens protein samples from different sets was calibrated at a concentration of 0.15 mg/ml in phosphate buffer (pH 7.4). For measuring the AGE fluorescence, the samples were

excited at 370 nm, while the fluorescence emission spectra was recorded between 400 and 500 nm using a Jasco make spectrofluorometer (FP-8300).

Measurement of glycation induced lens protein aggregates using NTA system

Nanoparticle Tracking and Analysis (NTA), LM20 system was employed for measuring the size of glycation induced lens protein aggregates (Filipe et al. 2010). The NTA LM20 operational settings were calibrated as per the manufacturer's manual. The glycated lens protein samples were diluted in ultrapure water to achieve the particle concentration of 10⁷–10⁹/ml. The calibrated protein samples were injected into LM 20 sample port using sterile syringes. After running the samples, the NTA software displays the results of particle size in terms of mean (average particle size measured) and mode (most frequently observed particle size) values.

Inhibition of aldose reductase (AR) using flavonoids

The preparation of semipure form of AR from goat lens and the AR inhibition assay was carried out as described previously (Gacche and Dhole 2011a). In brief, the reaction cocktail comprised of 1 ml of 50 μM potassium phosphate buffer (pH 6.2), 10 μM DL-glyceraldehyde, 0.1 μM NADPH, 0.4 mM lithium sulfate, 5 μM 2-mercaptoethanol and crude AR enzyme. The mixture was incubated at 37 °C for 10 min. The reaction was initiated by addition of NADPH and the fall in absorbance was recorded spectrophotometrically at 340 nm.

As a part of standardizing the experimental protocol, the test panel of flavonoids was initially tested over a wide range of concentrations and the range of 10–100 μM was selected to achieve the demonstrable AR inhibition. The percent inhibition with test flavonoids was calculated considering the AR activity in the absence of inhibitor as 100%. The concentration of each test flavonoids giving 50% inhibition (IC₅₀) was then calculated using nonlinear regression analysis. Quercetin, a known AR inhibitor has been used as a reference drug for comparison.

Molecular docking studies

For conducting computational molecular docking experiment, a well standardized protocol was utilized described in detail elsewhere (Gond et al. 2013; Kamble et al. 2016). Following steps briefly summarizes the procedure:

3D coordinates for the target protein (AR) co-crystallized with inhibitor zopolrestat in monomeric state having PDB ID 2DUX (Steuber et al. 2006), was obtained from the PDB database. At the initial stage, 2D depictions of flavonoids used in the current study were drawn in software ChemDraw

ultra. To obtain the 3D coordinates of these flavonoids in SDF format, their SMILES notation were saved in a separate file and uploaded on FROG server (Miteva et al. 2010). We utilized an open access software Python Prescription 0.8 (PyRx) interface for molecular docking purpose; wherein, AutoDock 4.2 (Morris et al. 1998), functions at background. PyRx has independent Open Babel module that was used to import the flavonoid ligands and convert them into the traditional pdbqt format. The grid box of $50 \times 50 \times 50$ points was placed around the inhibitor Zopolrestat to ensure that all residues from active site are included within the grid box. All default parameters were used during docking process.

Interactions of the flavonoids with the residues in the active site of aldose reductase were depicted in 2D plots using LigPlot⁺ (Laskowski and Swindells 2011). PyMol v0.99 was implemented to render the 3D molecular graphics images for docking.

Molecular dynamics (MD) studies

The MD simulation study was performed on AR docked complex with flavonoid ligands using GROMACS 5.0.7 program (Pronk et al. 2013). The Gromos53a6 force field and PRODRG server (Schüttelkopf and Van Aalten 2004), were employed in generation of the topology files for protein and ligand molecules, respectively. The complete system was then subjected to solvation using simple point charge (SPC) water model and Na⁺ counter ions were used to neutralize charges. The final system comprised of protein molecule, inhibitor, 14, 253 water molecules along with two Na⁺ ions. The steepest descent energy minimization method was employed for the minimization of the solvated structure. After energy minimization, a 200 ps equilibration simulation was performed in NVT ensemble applying position restraints over protein atoms. Finally, the system was equilibrated for time span of 1 ns using NPT ensemble. The final production run was performed for 10 nanoseconds (ns) at 300K temperature and constant pressure under isothermal-isobaric (NPT) ensemble. A 2 fs time step of was used throughout simulation with periodic boundary conditions. Berendsen et al. temperature (1987) and Parrinello and Rahman pressure coupling method (1981) were used to regulate the constant temperature and pressure of the system. To maintain the geometry of the molecule, the LINCS algorithm (Hess 2008) was used to constrain bond length. The PME algorithm was used in handling long range electrostatic interactions with cut off distance of 1.2 nm (Essmann et al. 1995). Majority of the analysis was carried out using inbuilt gromacs tool. The trajectory was analysed using VMD software and UCSF chimera (Humphrey et al. 1996; Pettersen et al. 2004). Cluster analysis was conducted to capture the representative structure from MD ensemble from each simulation on the basis of RMSD clustering with a threshold distance of 1.0 Å using

g cluster tool employed in GROMACS for further analysis. The images were rendered using Discovery Studio 3.5 and Chimera (Pettersen et al. 2004).

Statistical analysis

The experimental results summarized for various parameters are expressed as the mean of $n=4$, $\pm$ SD. The level of significance of the difference from the respective controls for each experimental test condition was calculated using Student's *t* test for individual experiment. A *p* value of <0.05 was considered as a significant difference.

Results and discussion

In the quest of understanding the role of dietary flavonoids in the amelioration of sugar induced cataractogenesis, recently we have analysed the sets of mono-, di-, and tri- hydroxylated flavonoids as putative modulators of sugar induced cataractogenesis. Our previous work has demonstrated the utility of 7-hydroxy flavone, chrysin, apigenin, baicalein, genistein, and rutin as lead molecules and scaffold for design and development of new and efficient compounds for the treatment of sugar induced cataractogenesis (Patil et al. 2016a, b; Patil and Gacche 2017). In the present investigation we have attempted to evaluate tetra-, penta- and hexa-hydroxylated panel of eight flavonoids (Supplementary Fig. 1) as inhibitors of glucose induced cataractogenesis.

Bovine lens organ culture study

The basic aim of the lens organ culture studies was to evaluate the influence of selected panel of flavonoids in maintaining lens transparency, structural integrity of lens and inhibition of protein aggregation in glycation induced cataract model studies. After incubation for 15 days, the images of the lenses exposed to various test flavonoids (except epigallocatechin gallate), clearly shows the efficacy of the test flavonoids in maintaining the lens transparency and structural integrity of the glycation induced cataractous lenses (Fig. 1). Of note, the effect of kaempferol, taxifolin and quercetin seems to be more impressive in this regard. The eye lens primarily function in focusing the light on the retina; therefore, the high refractive index and thereby the transparency is the basic prerequisite for visual acuity. Various key molecular mechanisms of flavonoids such as inhibition of oxidative stress, non-enzymatic glycation and polyol pathway have been attributed with anti-cataract effects in diabetic cataractogenesis (Stefek 2011; Patil et al. 2016a, b), however, the molecular mechanisms describing the role of flavonoids in maintaining lens transparency are poorly understood (Patil et al. 2016a). In vitro model settings, employing cultured rat

Fig. 1 Digitized representative images of the goat lenses showing effect of selected flavonoids (50 μ M) on glycation induced lens opacity, haziness, disruption and other morphological changes. The images of lenses were digitized after 15 days of incubation. Minimum four lenses (from two animals) were used in each set. **a** Negative con-

trol (KRB buffer + 30 mM glucose); **b** Positive control (Kreb Ringer Bicarbonate buffer); **c** aminoguanidine (reference drug); **F1** fisetin; **F2** luteolin; **F3** kaempferol; **F4** taxifolin; **F5** quercetin; **F6** catechin; **F7** myricetin; **F8** epigallocatechin gallate

lens revealed that low micromolar levels of quercetin inhibited oxidation mediated calcium and sodium influx and loss of lens transparency (Sanderson et al. 1999). Similar kind of results were described by Ramana et al. (2007), wherein the oral treatment of quercetin stabilized the disturbances of eye lens electrolytes such as calcium, sodium, potassium and maintained the lens protein levels along with preserving lens transparency. Rutin (a corresponding glycone of quercetin) has been shown to improve free radical mediated cataract by increasing the chaperone activity of α -crystallin (Sasikala et al. 2013). The results of these preclinical settings pinpoint the involvement of flavonoids in maintaining lens transparency by regulating the specific osmotic ion equilibrium and levels of lens proteins.

Determination of soluble and insoluble protein fractions of flavonoid treated lenses

After 15 days of incubation, the lenses from various sets were subjected for estimation of water soluble (WS) and insoluble (WIS) protein fractions. The results summarized

in Table 1, show that the lenses treated with kaempferol (95%), taxifolin (94%), myricetin (86%), quercetin (74%) and fisetin (73%) were found to possess significant amount of WS proteins in comparison with other test flavonoids which contained the WS fraction in a range of 52–68%. Of note, the results of kaempferol (95%) and taxifolin (94%) were at par with that of the reference drug aminoguanidine (94%). Cross linking of lens proteins coaxed by glycation is one of the contributing factors for accumulation of WIS fraction (Patil et al. 2016a). Apart from sustaining the natural tertiary structures of the lens proteins, the solubility of the lens crystallins is also instrumental in preserving the clarity of the eye lens (Sharma and Santhoshkumar 2009). The lenses containing significant amount of WS fraction has higher refractive index, while the reverse is true for the lenses containing considerable amounts of WIS fractions. Nevertheless, the aged and cataract lenses contains significant amount of WIS fractions which causes the scattering of light and thereby cause visual impairment (Sharma and Santhoshkumar 2009). Therefore, quantification of WS

Table 1 Profile of protein concentrations (soluble and insoluble) and size of protein aggregates in glycation induced lenses treated with different flavonoids

Sr. no	Flavonoids	Protein concentration (mg/gm of lens tissue)			Size of lens protein aggregates (nm)	
		Total lens protein (TP)	Lens soluble protein (TSP)	Soluble proteins (%)	Average size of lens protein aggregates (mean value)	Actual size of lens protein aggregates (mode value)
1	Positive control ^a	378 ± 6.8	348 ± 5.4	92 (08) ± 3.2	177 ± 3.6	135 ± 6.3
2	Negative control ^b	316 ± 7.2	169 ± 6.1	53 (47) ± 7.5	306 ± 3.9	287 ± 5.1
3	Aminoguanidine ^c	380 ± 5.3	358 ± 3.6	94 (06) ± 7.2*	221 ± 5.2	187 ± 4.0
4	Fisetin	388 ± 6.4	287 ± 4.8	73 (27) ± 6.4	205 ± 2.8	177 ± 4.6
5	Luteolin	312 ± 3.4	214 ± 4.7	68 (32) ± 5.7	176 ± 6.1*	168 ± 3.5
6	Kaempferol	600 ± 4.5	575 ± 3.8	95 (05) ± 4.8*	185 ± 4.5	150 ± 5.6*
7	Taxifolin	253 ± 5.7	240 ± 4.9	94 (06) ± 6.0*	166 ± 3.7*	144 ± 6.3*
8	Quercetin	222 ± 3.1	165 ± 2.8	74 (26) ± 4.2	173 ± 5.6*	159 ± 4.4*
9	Catechin	370 ± 8.5	240 ± 6.1	64 (36) ± 5.5	223 ± 4.1	196 ± 3.8
10	Myricetin	224 ± 6.0	193 ± 5.3	86 (14) ± 3.8*	210 ± 3.8	169 ± 3.3
11	Epigallocatechin gallate	300 ± 7.3	158 ± 6.2	52 (48) ± 5.6	215 ± 4.9	189 ± 7.1

The results summarized are the mean values $n=4$, $\pm$ SD

* $p < 0.05$ as compared to control

^aKRB Kreb Ringer Bicarbonate buffer

^bKRB buffer + 30 mM Glucose

^cAminoguanidine is a reference anti-glycation drug

^dValues in the parenthesis indicate the % of insoluble protein

and WIS fractions of lens may serve as a marker in the progression of cataractogenesis.

Estimation of protein carbonyl groups

The amount of carbonyl groups generated as result of glycation in individual treated and control set are summarized in Fig. 2. The results clearly indicate the efficacy of kaempferol (58 nmol/mg protein), taxifolin (69 nmol/mg protein) and quercetin (95 nmol/mg protein) as potential candidates for inhibition of sugar induced glycation of lens proteins as their treatment leads to generation of minimum amount of carbonyl groups in comparison to other tested flavonoids having carbonyl content in a range of 122–256 nmol/mg protein. As compared to reference drug aminoguanidine (48 nmol/mg protein), significant amount of carbonyl content was estimated from the lenses exposed to epigallocatechin gallate (256 nmol/mg protein) followed by catechin (217 nmol/mg protein). Glycation is an oxidation reaction wherein, the side chains of amino acids like arginine, threonine, lysine, and proline of proteins are more prone for undergoing sugar mediated oxidation, leaving the stable carbonyl groups at the oxidation site. The quantification of carbonyl groups provides cues about the extent of glyco-oxidative damage to lens proteins (Patil et al. 2016a). Amongst the tested flavonoids, extensive literature has been cited towards the impressive anti-cataract activities of quercetin, nevertheless

owing to its significant anti-glycation potential it has been described as a finer substitute to aminoguanidine in the inhibition of glycation and glycation end products (Ashraf et al. 2015), however, in our experimental settings, kaempferol and taxifolin demonstrated significant anti-glycation activity. Moreover, the comparative activity profile of the selected flavonoids in context with inhibition of generation of carbonyl groups is lacking in the previously cited literature.

Measurement of AGE fluorescence of glycated lens proteins

Figure 3 summarizes the results of the influence of selected flavonoids on the generation of AGEs. The results shown are the measurement of fluorescence of AGEs generated in the glycation induced lens tissue after 15 days of incubation. The results clearly focus the effectiveness of kaempferol > taxifolin > myricetin > luteolin > quercetin as potential inhibitors of AGEs formation evidenced with low fluorescence intensity. The samples treated with fisetin, epigallocatechin gallate and catechin did not demonstrate significant inhibition of AGEs formation. It has been proved that AGEs contributes significantly towards the formation of cataract by amending the charges on surface of the lens proteins and thereby inducing conformational changes which in turn adversely affect protein–protein and protein–water interactions and ultimately leads to reduction in the refractive

Fig. 2 Summary of amount of carbonyl groups generated in glycation induced bovine lenses treated with different flavonoids (50 µM). The results summarized are the mean values $\pm$ SD of four lenses

(derived from two animals) each used in individual four experiments. * $p < 0.05$ as compared to control

Fig. 3 Effect of selected flavonoids (50 µM) on glycation induced non-tryptophan AGEs fluorescence of lens proteins. The result summarized in figure is the representative image of individual four experiments performed with four lenses (derived from two animals)

index and transparency of the eye lens (Luthra and Balasubramanian 1993). Matsuda et al. (Matsuda et al. 2003) have described the structural requisites of flavonoids for inhibition of AGEs formation. The study affirms that the flavonoids with 3′-, 4′-, 5-, and 7-hydroxyl substituents demonstrate stronger AGEs inhibition. Moreover, the structural scaffold of flavones is recognized to be more active in AGEs inhibition in comparison to corresponding flavonols, flavanones, and isoflavones. To a greater extent, the results of the present studies are in concert with the structural requirements described. While describing the mechanism of action of AGEs inhibition using flavonoids, it has above been speculated that flavonoids interact with the intermediates of AGEs formation and thereby act as chain terminators. For example, quercetin has been reported to inhibit the AGEs intermediates such as methylglyoxal, 3-deoxyglucosone and glyoxal (Ashraf et al. 2015).

Measurement of glycation induced lens protein aggregates using NTA system

The measurements of the particle size of the glycation modulated lens protein aggregates are summarized in Table 1. The NTA LM 20 system (Fig. 4), which works on the basis of Stokes–Einstein equation was employed for the measurement of lens protein aggregates (Filipe et al. 2010). Minimum size of the protein aggregates were detected in lenses treated with taxifolin (144 nm). As compare to negative control set (287 nm) and the reference drug aminoguanidine (187 nm) the flavonoids like kaempferol (150 nm), quercetin (159 nm), luteolin (168 nm) and myricetin (169 nm) were also observed to be effective in avoiding the formation of protein aggregates after 15 days of glycation induced protein aggregation. The size of protein aggregates treated with remaining flavonoids was in a range of 177–189 nm. It is interesting to note that almost all test flavonoids maintained the protein aggregate size at par with that of reference drug aminoguanidine (187 nm).

It has been demonstrated that glycation bring about the serious age-related alterations in the membrane integrity of

Fig. 4 Representative image panel of effect of taxifolin (50 μ M) on glycation induced lens crystalline aggregate formation. The effect of selected flavonoids (50 μ M) on glycation induced lens crystalline aggregate formation was analyzed by measuring the sizes of lens protein aggregates using NTA LM 20 analysis system. **a** Plot representing particle size (nm) and concentration (particle/ml). Value 144

is mode size (in nm) of observed nano particles. **b** Still frame from video of nano sizing experiment. **c** Plot representing particle size (nm) and normalized intensity. **d** A 3D plot of particle size (10 nm per division) and relative intensity. The results summarized are the mean value of four lenses (derived from two animals) each used in individual four experiments

the lens fibre and structural levels of lens proteins, which ultimately leads to formation of aggregation and covalent crosslinking amongst the lens crystallin molecules causing reduced deformability with associated presbyopia regardless of cataract formation (Bron et al. 2000). Nevertheless, the levels of highly reactive AGEs such as glyoxal and methylglyoxal are elevated in diabetic condition which significantly contributes towards dense aggregate formation along with loss of chaperone activity of α -crystallin (Moreau and King 2012). The glycation triggered lens proteins aggregates is the main cause of light scattering and thereby loss of visual acuity. Flavonoids have been reported to possess protein disaggregation activity, especially against β -amyloid type protein aggregates. Bovine lens α -, β - and γ - crystallin are known to form amyloid type aggregated fibrils under specific denaturing conditions (Moreau and King 2012). Although the concrete molecular mechanism of flavonoid mediated protein disaggregation is not known, however, it has been projected that flavonoids acts by breaking chain during the process of aggregation thereby conferring stability to the re-folded proteins and disaggregates the pre-formed protein aggregates (Patil et al. 2016a). It is also speculated that the inhibitor might be directed to the amyloidogenic core via interactions between aromatic centres of flavonoid molecule with aromatic residues from the amyloidogenic sequence and thereby facilitate interaction, but inhibit the aggregate formation (Meng et al. 2010).

Inhibition of aldose reductase (AR) using flavonoids

The AR inhibitory activities of the selected flavonoids are shown in Table 2. The result clearly shows the significant AR inhibitory potential of luteolin (IC_{50} , 0.7 μ M), quercetin (IC_{50} , 1.8 μ M), taxifolin (IC_{50} , 3.2 μ M), fisetin (IC_{50} , 4.1 μ M) and kaempferol (IC_{50} , 6.5 μ M). Myricetin (IC_{50} , 26 μ M), epigallocatechin gallate (IC_{50} , 29 μ M) and catechin

(IC_{50} , 31 μ M) have also shown moderate AR inhibitory activity. It is known that in diabetes mellitus increased levels of glucose are associated with up regulation of AR activity and thereby increased levels of sorbitol in cells. Owing to slow metabolism of sorbitol by sorbitol dehydrogenase, it plays a crucial role in progression of cataractogenesis by adversely affecting the osmolarity of the lens tissue (Zhu 2013). It has been described that activation of aldose reductase triggers some molecular mechanism leading to excess accumulation of sorbitol, decrease in reduced glutathione, enhanced NADH/NAD ratio and enhanced oxidative stress (Jagdale et al. 2016). Furthermore, sorbitol generated gets metabolized to produce fructose that enters into the non-enzymatic glycation of proteins which is ultimately responsible for cataract development. Snow et al. (2015) showed that AR also induces the activation of some signaling molecules such as extracellular signal regulated kinase (ERK1/2) and c-Jun N-terminal (JNK1/2), which are already reported for its involvement in cell growth and apoptosis, respectively (Snow et al. 2015). Therefore, the AR inhibitory potential of the selected flavonoids can be explored in designing novel anti-cataract formulation owing to their dietary origin. While defining the structure activity relationship (SAR) of AR inhibition using flavonoids, it has been shown that the hydroxylation on A-ring of the flavone, particularly at 5th and 7th positions, expressively contributes towards AR inhibition. Hydroxylation at C3' and C4' positions of B-ring of flavonoids has remarkable effect on AR inhibition; however, the hydroxylation on the ring C of flavones has been shown to weaken the AR inhibitory potential. Moreover, the hydrogenation of the C2 = C3 double bond of flavones reduces the AR inhibitory potential (Xiao et al. 2015). To a greater extent our results are in line with the above described SAR.

Molecular docking studies

Almost all of the flavonoid candidates in this study except catechin and epigallocatechin gallate show a conserved polar interaction in form of hydrogen bonds with residues like Thr-113 and Leu-300. These residues are known to be critical players not only in catalysis, but also in inhibition of AR. For example, Thr-113 holds a key position in the "Specificity pocket" in AR. Numerous synthetic compounds like derivatives of Benzopyran-4-one and chalcones are experimentally characterized to result in opening the specificity pocket that leads to successful AR inhibition (Cousido-Siah et al. 2014). Various crystallographic evidences clearly demonstrate Thr-113 mediated, halogen bond induced inhibition of AR by inhibitor IDD388 (Podjarny et al. 2004). Artificial mutagenesis experiment conducted at this position demonstrate significant contribution towards maintaining thermodynamic stability of this enzyme; since, Thr113Ala mutants

Table 2 AR inhibitory activities of selected flavonoids

Sr. no.	Flavonoids	AR inhibition IC_{50} (μ M)
1	Fisetin	4.1 $\pm$ 0.8*
2	Luteolin	0.7 $\pm$ 0.1*
3	kaempferol	6.5 $\pm$ 1.2*
4	Taxifolin	3.2. $\pm$ 0.7*
5	Quercetin	1.8 $\pm$ 0.2*
6	Myricetin	26 $\pm$ 2.4
7	Catechin	31 $\pm$ 1.5
8	Epigallocatechin gallate	29.3 $\pm$ 1.5

The results summarized here are the mean values of three independent experiments, $\pm$ SD

* $p < 0.05$ as compared to control

are validated to deteriorate in serious binding free energy loss (Koch et al. 2011).

Highly conserved Leu-300 has been already characterized to stabilize AR- JF0064 complex by providing hydrophobic support. Experimental compound JF0064 is known to form polar interaction with backbone nitrogen of Leu-300 by its F3' atom (Cousido-Siah et al. 2014). Interestingly, this fact is in exact accordance with the molecular docking data obtained in this study. Besides these two conserved interaction, our molecular docking data demonstrate involvement of residue Cys-303 that is seldom disclosed in scientific literature in background of AR inhibition.

Catechin and epigallocatechin gallate are the only compound in the series that lack an oxo group on the fourth position in C ring of flavone scaffold and hence lack 2–3 double bond. This factor might have allowed catechin and epigallocatechin gallate to take different orientation in active site and hence forms hydrogen bonding pattern distinct than rest of the flavonoids that have similar hydrogen bonding pattern (Fig. 5). Differential orientation of catechin and epigallocatechin gallate can be observed as a main reason that enabled these flavonoids to make hydrogen bonding with

new residues like Trp-111, Ser-159, Asn-160, Cys-298, and Ala-299. These hydrogen bonds are not observed with rest of the flavonoids. However, this orientation does not appear to make a significant contribution in defining binding free energy of compound towards AR active site. In fact the binding free energy appears to be decreased with absence of oxo group. Thus, truancy of oxo group can be considered as an important factor that determines the thermodynamic stability of flavonoids in AR active site. Moreover, presence of 2–3 double bond is considered as a key determinant of bioactivity in case of flavonoids (Gacche et al. 2015). This preposition is again supported by in vitro experimental observations from current study that catechin and epigallocatechin gallate has higher IC_{50} values in comparison with remaining compounds.

Molecular dynamics (MD) studies

Molecular docking is a widely accepted technique to infer structural interactions in the protein and inhibitor complexes. However, since it provides static picture of the protein inhibitor complex, many interactions occurring due to

Fig. 5 Docking results of promising flavonoids (yellow sticks) in aldose reductase (cartoon representation). Residues forming hydrophobic/van der Waals contacts are represented in blue lines. Residue

atoms forming hydrogen bonds (magenta lines) with flavonoid molecules are shown as cyan sticks. **a** Leutolin, **b** quercetin, **c** catechin, **d** epigallocatechin gallate

conformational changes in protein owing to the dynamic nature of biological systems are missed in docking analysis. Moreover, catalysis as well as inhibition process of AR is reported to be involving conformational changes. For example, Borhani et al. (1992), suspected conformational rearrangement of loop-7 during catalysis and residues from the specificity pocket are known to undergo dynamic orientations leading to AR inhibition (Zhang et al. 2013). In view of the fact that very little is known about these dynamic behaviour, we initiated separate molecular dynamics simulation of the selected four flavonoids-AR complexes (on the basis of experimental IC_{50} values) to monitor conformational changes when AR interacts with flavonoid molecules.

The root mean square deviation (RMSD) is generally implemented in determination of convergence of protein-ligand system. To demonstrate stability of AR molecule upon flavonoid binding, we estimated the RMSD profile. The initial frame was considered as reference structure in RMSD calculations of protein's backbone atoms. Time evolution of AR-Flavonoid complex for span of 10 ns is depicted in Supplementary Fig. 2. The trend observed in Supplementary Fig. 2A clearly represent an initial burst in RMSD that stabilized between 0.15 and 0.2 nm during initial 3 ns and subsequently, a plateau stage is attained that displays very little and acceptable drifts in RMSD values in case of luteolin, quercetin, taxifolin. This plateau remains permanent till the end of the simulation. Such conformational behaviour reflects system stability. It is to be noted that Fisetin-AR complex attained convergence at higher RMSD levels as compared to remaining three studied flavonoids. Such a trend is reported to indicate enhanced stability of remaining compounds like luteolin, quercetin and taxifolin with AR as compared to fisetin (Cui et al. 2015).

RMSF is often implemented to understand local structural flexibility in a molecule. The highest RMSF values were recorded for the loop region and the core structural fold regions in proteins are generally rigid and hence residues in this region were observed to have minimum flexibility. The Supplementary Fig. 2B clearly indicate larger extent of fluctuation of C α atoms around two regions Phe-121 to Asn-136 and Pro-211 to Ser-226 (loop-7). The region corresponding to Phe-121 to Asn-136 most important residues that regulate the specificity pocket and hence it is necessary to effective catalysis as well as inhibition of AR. Thus, the increased flexibility in these loop regions reflects effective opening and closing of specificity pocket to accommodate flavonoid molecules. Loop-7 contains residue like Trp-219 whose structural flexibility is already known to govern fluorescent properties of AR (Rondeau et al. 1992). Thus, our MD simulation data are in exact line with experimental observation of Borhani et al. (1992) that demonstrate that B factors of residues in this loops are reported to have high thermal vibration values that are crucial for inhibition of AR.

Lys-221 and Leu-301 are structurally located at tip of loops (loop-7 and loop-8, respectively) that defines open and closed states of AR. Plot of distance between these two residues thus corresponds to opening-closing phenomenon of enzyme AR. Trend observed in Supplementary Fig. 3A clearly indicate closing the enzyme upon forming complex with fisetin and quercetin. Simultaneously, the distance plot between residue Trp-219 and Trp-295 (Supplementary Fig. 3B) also demonstrates the trend of increased distance during the course of simulation. These facts evidently conclude that upon flavonoid binding, the distance between the two residues (Trp-219 and Trp-295) gradually increases with closing the loop. These results are in agreement with experimental fluorescence results data from Rondeau et al. (1992). Borhani et al. (1992) suspected involvement of residues Arg-293, Pro-218, and Arg-217 which function to confiscate Trp-219 and Trp-295 from solvent in open state. It was postulated that, upon loop closing, the increased distance results in solvation of these tryptophans and thus facilitating effective fluorescence quenching.

It was hypothesised that the inter-conversion of AR enzyme between activated and inactivated form is mediated by oxidation and reduction, respectively; while, activation is dependent on disulphide bond formation between cysteine thiol groups (Vander Jagt et al. 1990). Moreover, biochemical mutagenesis experiment conducted at position Cys-298 demonstrated that resulting Ser-298 mutant leads to deteriorated enzyme kinetics (Kubiseski et al. 1992). Elementary, structural analysis of the AR model indicate that Cys-80 and Cys-303 are the only two cysteine residues that have thiol groups oriented towards Cys-298 and may potentially form disulphide linkages. The initial distance of Cys-80 and Cys-303 from Cys-298 is found to be approximately 11 Å. Plotting the distance between thiols of these residues along the course of simulation may clarify the possibility of formation of disulphide linkages. Distance plot of side chain thiols of Cys-298 and Cys-80 clearly demonstrate gradual increase in distance between thiol groups in case of fisetin, luteolin, and taxifolin. Slight decrease in distance is observed in case of quercetin; but again, it does not qualify for minimum distance for formation of a disulphide bond. Similarly, in case of Cys-303 and Cys-298 plot, luteolin along with quercetin demonstrate slight drop in thiol distances but cannot qualify to form a disulphide linkage (Supplementary Fig. 4). In view of the above mentioned facts, very low chances of formation of disulphide linkage are observed in current study. Thus, molecular dynamics data from current investigation is in accordance with Borhani et al. (1992), that proposed activation of AR may not involve formation of disulphide bridge.

In order excerpt the best and distinct structural features derived from separate four MD simulations, we employed cluster analysis approach. Cluster analysis data for specificity pocket clearly demonstrate similar binding mode and

Fig. 6 Left panel: Bound conformation of inhibitor (yellow) **a** fisetin, **b** leutolin, **c** quercetin, **d** taxifolin to ar specificity pocket with important interacting residues shown in stick representation. Right panel: 2D interaction of inhibitor with AR specificity pocket

Table 3 Summary of the virtual screening and structural analysis data of various flavonoids in complex with enzyme aldose reductase

Ligand	Binding free energy (Kcal/mol)	Hydrogen bonding pair (HBD in Å) (HD::HA)	Residues in hydrophobic interaction or van der Waals contact
Fisetin (3,3',4',7-tetrahydroxy flavone)	− 8.39	Lig (A) C7 ^{OH} ::Thr113(O ^{sc}):OG1(2.82) Leu300(N ^{bb})::Lig(C):C4 ^{Ox} (2.67) Lig(A) C7 ^{OH} ::Cys303 (S ^{sc}):SG(3.11)	Trp20, Tyr48, Trp79, Trp111, Phe115, Phe122, Cys298, Ala299
Luteolin (3',4',5,7-tetrahydroxy flavone)	− 8.94 ^a	Lig(A)C7 ^{OH} ::Thr113(O ^{sc}):OG1 (2.83) Leu300(N ^{bb})::Lig(C):C4 ^{Ox} (2.89) Lig(A):C5 ^{OH} ::Leu300(N ^{bb}):N (2.93) Lig(A):C7 ^{OH} ::Cys303 (S ^{sc}):SG (3.10)	Trp20, Tyr48, Trp79, Trp111, Phe115, Phe122, Cys298, Ala299
Kaempferol (3,4',5,7-tetrahydroxy flavone)	− 8.62	Lig(A):C7 ^{OH} ::Thr113(O ^{sc}):OG1(2.73) Leu300(N ^{bb})::Lig(C):C4 ^{Ox} (2.82) Lig(A):C5 ^{OH} ::Leu300(N ^{bb}):N (3.06) Lig(A):C7 ^{OH} ::Cys303 (S ^{sc}):SG (3.27)	Trp20, Tyr48, Trp79, Trp111, Phe115, Phe122, Cys298, Ala299
Taxifolin (3,3',4',5,7-pentahydroxy flavonone)	− 8.31	Lig(A):C7 ^{OH} ::Thr113(O ^{sc}):OG1(2.92) Leu300(N ^{bb})::Lig(C):C4 ^{Ox} (2.94) Lig(A):C5 ^{OH} ::Leu300(N ^{bb}):N (2.93) Lig(A):C7 ^{OH} ::Cys303 (S ^{sc}):SG (3.04)	Trp20, Tyr48, Trp79, Trp111, Phe115, Phe122, Cys298, Ala299
Quercetin (3,3',4',5,5',7-pentahydroxy flavonol)	− 8.38	Lig(A):C7 ^{OH} ::Thr113(O ^{sc}):OG1(2.88) Leu300(N ^{bb})::Lig(C):C4 ^{Ox} (2.79) Lig(A):C5 ^{OH} ::Leu300(N ^{bb}):N (2.92) Lig(A):C7 ^{OH} ::Cys303 (S ^{sc}):SG (3.07)	Trp20, Val47, Tyr48, Trp79, Trp111, Phe115, Phe122, Cys298, Ala299, Tyr309
Myricetin (3,3',4',5,5',7-hexahydroxy flavone)	− 7.89	Lig(A):C7 ^{OH} ::Thr113(O ^{sc}):OG1(2.96) Leu300(N ^{bb})::Lig(C):C4 ^{Ox} (3.15) Lig(A):C5 ^{OH} ::Leu300(N ^{bb}):N (2.89) Lig(A):C7 ^{OH} ::Cys303 (S ^{sc}):SG (2.94)	Trp20, Val47, Tyr48, Trp79, Trp111, Phe115, Phe122, Cys298, Ala299
Catechin [(−)-trans-3,3',4',5,7-pentahydroxy flavonone]	− 7.74	Lig(C):C7 ^{OH} ::Trp111(N ^{sc}):NE1 (2.82) Lig(B):C3 ^{OH} ::Ser159(O ^{sc}):OG (3.10) Lig(B):C3 ^{OH} ::Asn160(N ^{sc}):ND2 (3.09) Cys298(S ^{sc}):H::Lig(C):O1(2.72) Lig(A):C3 ^{OH} ::Ala299(N ^{bb}):N(2.88)	Trp20, Tyr48, Trp79, His110, Phe122, Tyr209, Trp219, Leu300
Epigallocatechin gallate [(−)-cis-3,3',4',5,5',7-hexahydroxy flavonone]	− 7.15	Lig(C):C7 ^{THB} ::Val47(O ^{bb}):O (2.53) Lig(A):C3 ^{OH} ::Tyr48(O ^{sc}):OH (3.09) Lig(C):C7 ^{THB} ::Gln49(N ^{sc}):NE2 (3.17) Lig(A):C3 ^{OH} ::His110(N ^{sc}):NE2 (2.83) Lig(B):C3 ^{OH} ::Leu300(N ^{bb}):N (2.94)	Trp20, Trp79, Trp111, Phe121, Phe122, Trp219, Cys298, Ala299

The interaction Lig(A)C7^{OH}::Thr113(O^{sc}):OG1 is to be inferred as: Hydroxyl group at C7 position in A ring of flavonoid ligand hydrogen bonds with OG1 atom of side chain oxygen of Threonine113

Lig ligand, sc side chain, bb back bone, Ox oxo oxygen, THB trihydroxybenzoate, HB hydrogen bond, HBD hydrogen bond distance, Å Angstrom, HD hydrogen donor group, HA hydrogen acceptor group

^aFlavonoid compounds showing best binding free energy value towards Aldose reductase

orientation of flavonoids in all four representative structures obtained after MD simulation. Furthermore, AR residues are observed to establish similar interaction pattern in all complexes with some minor differences. For example, residues Trp-111 and Trp-79 are found to be engaged in pi–pi staking interaction with fisetin, luteolin, and quercetin; while, staking interaction of Trp-79 is replaced with Trp-20 in taxifolin complex (Fig. 6). In line with previous study, we observed that the side chain of Trp-111 orients and acquire B10 position which might facilitate the accommodation of bulky chromen-one moiety of flavonoids in specificity pocket (Zhang et al. 2013). This observation thus explains a specific mode of flavonoid based AR inhibition. The residue Cys- 303 and Leu-300 illustrate pi-T

shaped interaction with inhibitor except taxifolin (Fig. 6). Pi-sulfur interaction was also evidently noted during MD simulation of selected flavonoids. For instance fisetin and quercetin are found to be in pi-sulfur contact with residue Cys-80. Luteolin appears to establish pi-sulfur interaction with Cys-300, whereas taxifolin did not appear to form any such interaction. Interestingly, such sulphur–aromatic interactions are experimentally known to play an imperative role in stabilization of the closed form of enzyme and ultimately limit the rate of catalysis of aldehyde reduction reaction (Grimshaw et al. 1995a, b). It is noteworthy that MD simulation revealed hydrogen bonding of flavonoids with residues like Trp-20, Cys-80 and Ser-302 (Fig. 6) that were not evident in molecular docking results (Table 3).

These residue interaction networks provide stability to bulky side chain of inhibitor in specificity pocket.

Conclusion

In the present study, we demonstrate the efficacy of the selected flavonoids as potential candidates for controlling the diabetic cataract. The outcomes of the present investigation also focus the significance of kaempferol, taxifolin and quercetin as potential candidates for management of glycation induced cataractogenesis. Molecular docking data described in this paper focuses on important interactions that have enormous possibilities to be explored in design and development of new flavonoid based anti-diabetic agents. Structural movement of various loops and residues from specificity pocket discussed here sheds light on dynamic behaviour of flavonoid based AR inhibition. The results of the present study has potential to find applications in developing a novel and effective formulation of functional foods or nutraceuticals from dietary sources rich in selected flavonoids, perhaps will serve an alternative and complementary therapy for the effective management of diabetic cataract.

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Compliance with ethical standards

Conflict of interest All contributing authors declare no conflicts of interest.

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