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Emblica officinalis and its enriched tannoids delay streptozotocin-induced diabetic cataract in rats

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Purpose: Aldose reductase (AR) has been a drug target because of its involvement in the development of secondary complications of diabetes including cataract. We have previously reported that the aqueous extract of *Emblica officinalis* and its constituent tannoids inhibit AR in vitro and prevent hyperglycemia-induced lens opacification in organ culture. The purpose of the current study was to investigate the effect of Emblica and its enriched tannoids on streptozotocin (STZ)-induced diabetic cataract in rats.

Methods: Diabetes was induced in Wistar-NIN rats by STZ (35 mg/kg body weight, intraperitoneally) and the animals were divided into three groups (Group II, III, and IV). The control rats (Group I) received only vehicle. While Group I and Group II animals received AIN-93 diet, rats in Groups III and IV received 0.2% of standardized mixture of Emblica tannoids and 2% of Emblica pericarp, respectively, in an AIN-93 diet for a period of eight weeks. Cataract progression due to hyperglycemia was monitored by slit-lamp biomicroscope and classified into four stages. At the end of the eight weeks, the animals were sacrificed and markers of the polyol pathway, oxidative stress, and alterations in protein content and crystallin profile in the lens were measured. Blood glucose and insulin levels were also determined.

Results: Both Emblica and its tannoids did not prevent STZ-induced hyperglycemia as assessed by blood glucose and insulin levels. However, slit lamp microscope observations indicated that these supplements delayed cataract progression. The present studies suggest that Emblica and its tannoids supplementation inhibited AR activity as well as sorbitol formation in the lens. The results also point out that Emblica and its tannoids might counter the polyol pathway-induced oxidative stress as there was a reversal of changes with respect to lipid peroxidation, protein carbonyl content, and activities of antioxidant enzymes. Emblica also prevented aggregation and insolubilization of lens proteins caused by hyperglycemia.

Conclusions: The results provide evidence that Emblica and an enriched fraction of Emblica tannoids are effective in delaying development of diabetic cataract in rats.

Diabetes mellitus is a heterogeneous metabolic disorder characterized by high levels of blood glucose. Prolonged exposure to uncontrolled chronic hyperglycemia in diabetes can lead to various complications in the eye including cataract and retinopathy [1,2]. Cataract, characterized by cloudiness or opacification of the eye lens, is the leading cause of blindness all over the world. In view of the widespread prevalence of diabetes in developing countries like India [3-5], diabetic cataract may pose a major problem in the management of blindness. Although the pathogenesis of diabetic complications is not known, many biochemical pathways associated with hyperglycemia have been implicated [1]. Among these, the polyol pathway has been extensively studied. Aldose reductase (AR) is the first and rate-limiting enzyme of the polyol pathway [6]. Under euglycemic conditions, AR plays a minor role in glucose metabolism; however, during diabetes, its contribution is significantly enhanced [7,8] leading to a conversion of excess glucose to sorbitol in insulin independent tissues like the lens. AR dependent synthesis of excess polyols has been implicated as one of the mechanisms leading to diabetic and galactosemic cataracts [9-12].

A number of studies with experimental animals suggest that the compounds that inhibit AR could be effective in the prevention of certain diabetic complications [13-15]. To date, a number of AR inhibitors (ARI) such as tolrestat, epalrestat, zenarestat, zopolrestat, and sorbinil have been found to improve some diabetic complications in animal experiments and have been developed to the point of clinical evaluation. Nonetheless, clinical trials of ARI against neuropathy and retinopathy have met with limited success and some of the synthetic ARI were associated with deleterious side effects and poor penetration of target tissues such as the nerve and retina [16,17].

Therefore, in recent years, there is increased interest in identifying natural sources of ARIs that can be tested for their therapeutic value against diabetic complications [18-20]. In this context, we have been investigating the potential of spice/dietary sources which include Emblica to prevent diabetic cataract in animals [21-24]. *Emblica officinalis* Gaertn., commonly known as *amla*, is extensively used in many preparations of Ayurveda (one of the systems of Indian traditional medicine) and also against many chronic ailments including diabetes [25-27]. We have previously reported that the aqueous extract of Emblica inhibited AR and showed that the hydrolysable tannoids present in Emblica are responsible for the inhibition [22]. Furthermore, we also showed that tannoids of Emblica prevented sugar-cataract in a lens organ culture system [22].

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These results imply that constituents of *Embllica* may be explored as potential therapeutic agents against diabetic cataract. In the present study, we evaluated the efficacy of whole *Embllica* pericarp and the enriched tannoid mixture for effectiveness in prevention or delay in the onset and progression of cataracts in the streptozotocin (STZ)-induced diabetic rat model.

METHODS

Materials: STZ, NADPH, NADH, 2-thiobarbituric acid (TBA), 1,1,3,3-tetraethoxy propane (TEP), DL-glyceraldehyde, lithium sulfate, β -mercaptoethanol, glutathione, glutathione reductase, BSA, 2,4-dinitrophenylhydrazine (DNPH), and EDTA were obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals and solvents were of analytical grade and were obtained from local companies.

Preparation of *Embllica* pericarp powder: Fresh fruits of *Embllica* were collected from September through October from the local market. The pericarp of the fruit was freeze-dried. The dried material was powdered and used for the experiment by mixing it with an AIN-93 diet.

Standardized mixture of *Embllica* tannoids: Four hydrolysable tannoids, emblicanin A, emblicanin B, punigluconin, and pedunculagin have been isolated from *Embllica* pericarp and their structures have been established [28]. We have obtained the enriched fraction of *E. officinalis* juice with the above tannoids as a standardized extract in the powder form gratis from Indian Herbs Research & Supply Company, Saharanpur, India. The relative proportions of different tannoids in the standardized extract are as follows: emblicanin A and B, 35-55%; punigluconin, 4-15%; pedunculagin, 10-20%; rutin, 3%; and gallic acid, 1%.

Experimental design: Male, WNIN rats (two to three months old) with an average body weight of 231 ± 11 g (obtained from the National Center for Laboratory Animal Sciences, National Institute of Nutrition, Hyderabad, India) were used in the study. Diabetes was induced in overnight-fasted animals by a single intraperitoneal injection of STZ (35 mg/kg) in 0.1 M citrate buffer, pH 4.5. Another set of rats, which received only vehicle, served as the control (Group I; n=8). Fasting blood glucose levels were measured 72 h after STZ injection. Animals having blood glucose levels >145 mg/dL were considered diabetic and were divided into three groups (Group II, III, and IV). Animals in these groups received either only the AIN-93 diet (Group II; n=13) or received the AIN-93 diet containing 0.2% tannoids mixture (Group III; n=9) or 2.0% of *Embllica* powder (Group IV; n=9). *Embllica* contains 10-12% of tannoids and hence 0.2% tannoids corresponds to approximately 2% *Embllica*. Animals were treated as described for a period of eight weeks and were housed in individual cages in a temperature- and humidity-controlled room with a 12 h light-dark cycle. All animals had free access to water. Food intake (daily) and body weights (weekly) were monitored during the experiment. Animal care and protocols were in accordance with and approved by the Institutional Animal Ethics Committee and conformed to the ARVO State-

ment for the Use of Animals in Ophthalmic and Vision Research.

Slit lamp examination and cataract classification: Eyes were examined every week using a slit lamp biomicroscope (Kowa Portable, Japan) on dilated pupils. Initiation, progression, and maturation of lenticular opacity was graded into five stages as follows: stage 0 - clear lenses and no vacuoles present; stage 1 - vacuoles cover approximately one-half of the surface of the anterior pole forming a sub capsular cataract; stage 2 - some vacuoles have disappeared and the cortex exhibits a hazy opacity; stage 3 - a hazy cortex remains and dense nuclear opacity is present; stage 4 - a mature cataract is observed as a dense opacity in both cortex and nucleus [23].

Blood, lens collection, and processing: Blood was collected once a week from the retroorbital plexus for glucose and insulin estimation. At the end of eight weeks, the animals were sacrificed by CO₂ asphyxiation and the lenses were dissected by the posterior approach and stored at -85 °C until further analysis. A 10% lens homogenate was prepared from three to five pooled lenses in a 50 mM phosphate buffer, pH 7.4. All the biochemical parameters were analyzed in the soluble fraction of the lens homogenate (15,000x g at 4 °C) except for lens malondialdehyde (MDA) and sorbitol, those were determined in the total homogenate.

Clinical parameters: Serum glucose and insulin were measured by the glucose oxidase-peroxidase method with a commercial kit (Ozone Biomedicals Pvt. Ltd., New Delhi, India) and by an RIA kit (BRIT-DAE, Mumbai, India), respectively.

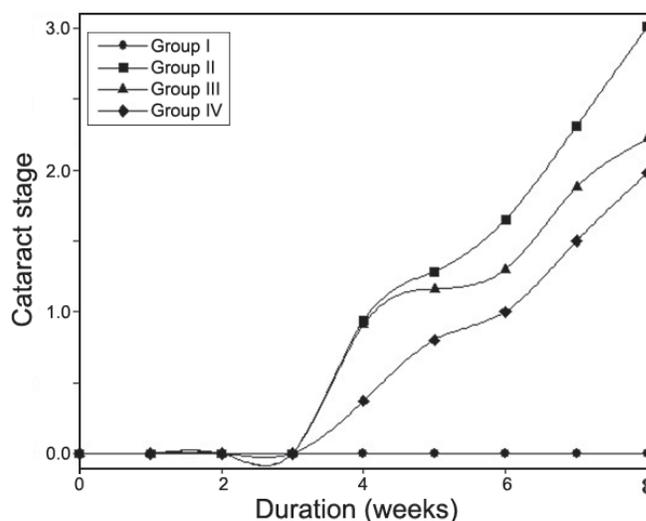


Figure 1. Delay of diabetic cataract in rats by *Embllica* and its tannoid-enriched fraction. Cataract formation was monitored weekly by slit-lamp microscope and the stage of cataract was scored according to the classification described in the Methods section. Stages of cataract in each group were averaged at the given time and the average stage of cataract was plotted as a function of time. *Embllica* (Group IV) and its constituent tannoids (Group III) delayed the maturation of diabetic cataract due to slow progression compared to untreated diabetic rats (Group II). Lenses in control rats (Group I) were clear during the experimental period.

Biochemical estimations: Lens MDA was measured as thiobarbituric acid reacting substances (TBARS) [29] and protein carbonyl content was determined according to reported methods [30]. The activities of AR [22] and sorbitol dehydrogenase (SDH) [31], superoxide dismutase (SOD) [32], glutathione peroxidase (GPx) [33], and glutathione S-transferase (GST) [34] were assayed according to the reported methods. Sorbitol levels in the lens were estimated by enzymatic method [35]. Total and soluble protein was analyzed by the Lowry method with bovine serum albumin as a standard.

SDS-PAGE and size exclusion chromatography of lens proteins: Subunit profile and cross-linking of soluble proteins were analyzed by SDS-PAGE under reducing conditions. Crystallin distribution in the soluble protein fraction was evaluated by size exclusion chromatography according to previously reported methods [23].

Data analysis: One-way ANOVA was used for testing statistical significance between groups of data and individual pair difference was tested by means of Duncan's multiple-range test. Heterogeneity of variance was tested by the non-parametric Mann Whitney test. A $p < 0.05$ was considered significant.

RESULTS & DISCUSSION

Food intake and body weights: There was an increase in the food intake in all the diabetic groups (II, III, and IV) compared with the control group (data not shown). Despite the increased food intake, the body weight of diabetic (Group II) animals was decreased when compared with nondiabetic controls (Group I). However, feeding of either tannoids or Emblica to diabetic rats (Group III and IV) did not normalize body weights to a significant extent (data not shown).

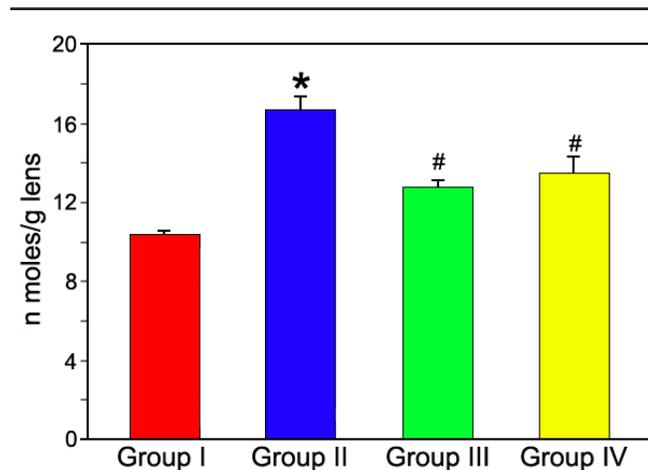


Figure 2. Effect of Emblica and tannoids on lipid peroxidation in the lens. Lipid peroxidation was assessed by estimation of TBARS in the total lens homogenate. TBARS levels, a measure of lipid peroxidation, increased in untreated diabetic (Group II) compared with control (Group I). Emblica (Group IV) and its tannoids (Group III) prevented the alterations in TBARS. Data are mean \pm SD (n=4). The asterisk above the bar denotes that data are significantly different from Group I and the sharp (hash mark) denotes that data are significantly different from Group II.

Cataract progression: The onset of cataract due to hyperglycemia was observed in diabetic animals after four weeks of STZ injection. The average incidence of cataract was calculated and presented in Figure 1. Interestingly, there was a delay in the onset of cataract in Group IV animals when compared to Group II. However, such a delay was not observed in Group III animals. This observation indicates that the whole Emblica was more effective than its tannoid fractions in delaying the onset of cataract. At the end of eight weeks, the severity of cataracts was significantly lower in groups III and IV than in Group II, indicating that the Emblica and its constituent tannoids delayed the maturation of diabetic cataract due to slow progression. All the lenses in Group I during the experiment period appeared to be normal and free of opacities

TABLE 1. THE EFFECT OF EMBLICA AND TANNOIDS ON MARKERS OF THE POLYOL PATHWAY IN LENS

Enzyme	Group I	Group II	Group III	Group IV
AR	24.96 \pm 1.04	30.98 \pm 1.27*	27.14 \pm 0.16#	27.55 \pm 1.69#
SDH	3.59 \pm 2.07	3.57 \pm 1.00	3.35 \pm 1.02	3.12 \pm 1.09
Sorbitol	0.24 \pm 0.02	5.74 \pm 0.77*	3.63 \pm 0.47#	3.92 \pm 0.27#

Emblica (Group IV) and tannoids (Group III) are partially effective against osmotic stress caused by hyperglycemia (Group II) with respect to AR activity and sorbitol levels. Group I: Control rats. The data are the mean \pm SD (n=4). AR activity was expressed as μ moles NADPH oxidized/h/100 mg protein. SDH activity was μ moles NADH oxidized/h/100 mg protein. Sorbitol was expressed as μ moles/g lens. The asterisk denotes that data are significantly different from Group I and the sharp (hash mark) denotes that data are significantly different from Group II.

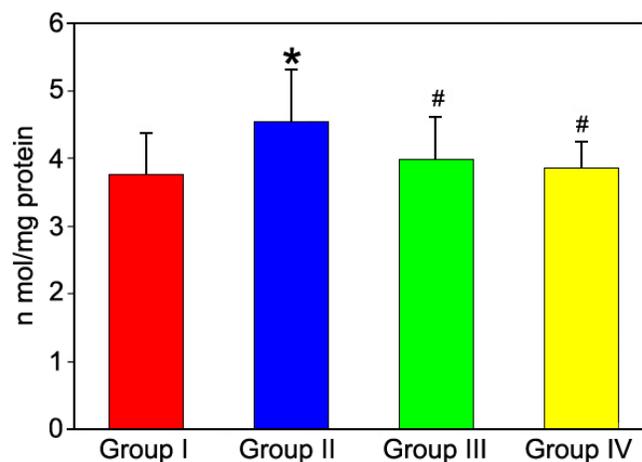


Figure 3. Protein carbonyl content in the soluble protein fraction of lens. Protein carbonyl groups of soluble protein fraction of different groups were assayed by reactivity to 2,4-DNPH as described in the Methods section. Protein carbonyl content, a measure of oxidative damage to proteins, increased in untreated diabetic (Group II) compared with controls (Group I). Emblica (Group IV) and its tannoids (Group III) prevented the alterations in protein carbonyls. Data are mean \pm SD (n=4). The asterisk above the bar denotes that data are significantly different from Group I and the sharp (hash mark) denotes that data are significantly different from Group II.

(Figure 1) as were lenses from nondiabetic rats treated with either Emblica or tannoids (data not shown). These data suggest that progression of lens abnormalities and maturation of cataracts due to STZ-induced hyperglycemia were significantly delayed with feeding of Emblica and its tannoids. The effect was slightly more pronounced with Emblica treatment.

Blood glucose and insulin: Blood glucose was measured weekly and insulin levels were measured at the end of the experiment to test whether Emblica and its tannoids had a direct effect on STZ-induced hyperglycemia. As expected, blood glucose levels were elevated and insulin levels were decreased significantly in Group II compared to Group I [23]. However, treatment with either Emblica or its tannoids did not reverse the changes in blood glucose and insulin levels, indicating that the Emblica and tannoids treatment had no effect on the hyperglycemia (data not shown).

Polyol pathway: While the specific activity of AR, a key enzyme of the polyol pathway, was significantly higher in Group II than in Group I, SDH activity was not significantly altered (Table 1). AR activity in lenses from animals treated with Emblica and tannoids were intermediate to Groups I and II, consistent with our previous observations on the in vitro inhibition of human recombinant and rat lens AR by Emblica tannoids [22]. Further, there was an increase of sorbitol levels in Group II animals when compared to Group I (Table 1), which was expected following activation of the polyol pathway in diabetic lens. However, feeding of tannoids and Emblica resulted in a lower but incomplete normalization of diabetes-induced sorbitol accumulation (Table 1). Based on these results, it appears that Emblica and tannoids are partially effective against osmotic stress caused by hyperglycemia. These results are consistent with the hypothesis that the delay of diabetic cataract by Emblica and its tannoids is related to inhibition of AR and of accumulation of sorbitol in the lens. Moreover, partial inhibition of AR and sorbitol accumulation brought about by Emblica and its tannoids is in agreement with a delay and not a complete prevention of diabetic cataract.

Oxidative stress and the antioxidant system: There is accumulating evidence that oxidative stress contributes to the development of diabetic cataract [12,36-38]. Recent studies indicate that the polyol pathway may be related to hypergly-

cemia-induced oxidative stress and there may be a metabolic connection between the polyol pathway and oxidative stress [12,39,40]. It was also reported that AR inhibitors reduce oxidative damage [12,40]. In the present study, we assessed oxidative stress by measuring TBARS, protein carbonyls, and some of the antioxidant enzymes in the lens. Increased TBARS levels in the untreated diabetic group (Group II) compared to nondiabetic controls (Group I) indicate increased lipid peroxidation in the lens due to hyperglycemia (Figure 2). Protein carbonyl content, a measure of oxidative damage to proteins, was also found to be increased in Group II compared to Group I suggesting enhanced protein oxidation under hyperglycemic conditions (Figure 3). Interestingly, treatment with

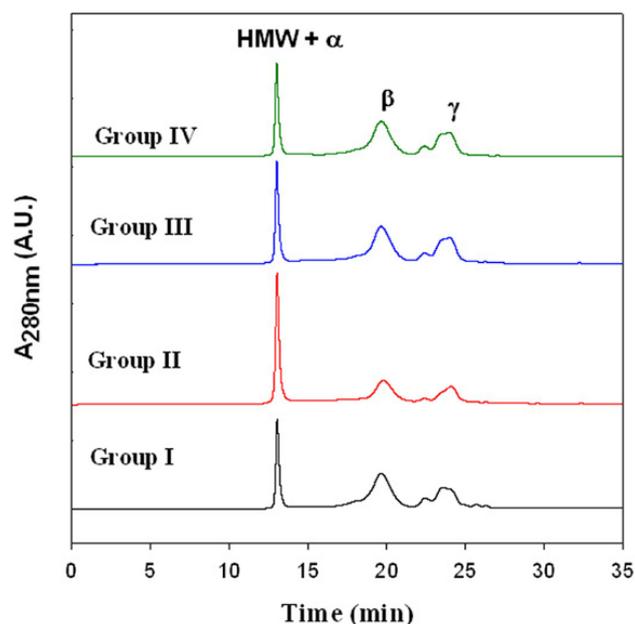


Figure 4. The distribution profile of crystallins in the soluble fraction of lens. The distribution profile of crystallins in the soluble fraction of lens of different groups was monitored by size exclusion chromatography (SEC). Soluble protein (20 μ l of a 1 mg/ml dilution in equilibration buffer) was loaded on TSK-G 2000 SW SEC column and protein peaks were detected at 280 nm with a flow rate of 1 ml/min. Peaks representing α -, β -, and γ -crystallins and high molecular weight (HMW) fractions are indicated at their respective positions.

TABLE 2. ACTIVITIES OF SUPEROXIDE DISMUTASE, GLUTATHIONE PEROXIDASE, AND GLUTATHIONE S-TRANSFERASE IN LENS

Enzyme	Group I	Group II	Group III	Group IV
SOD	26.81 \pm 0.62	36.88 \pm 6.95*	32.15 \pm 7.65#	26.84 \pm 0.67#
GPx	17.31 \pm 0.52	18.42 \pm 0.75*	17.84 \pm 0.40#	18.77 \pm 0.20
GST	24.82 \pm 0.55	22.18 \pm 0.48*	22.11 \pm 1.86	23.37 \pm 0.35

Emblica (Group IV) and tannoids (Group III) treatment partially prevented the altered activities of antioxidant enzymes in untreated diabetic group (Group II). Group I: Control rats. The data are the mean \pm SD (n=3). SOD activity is expressed as units/min/100 mg protein. The activity of GPx and GST is expressed as μ moles of NADPH oxidized/h/100 mg protein and mmoles of CDNB-GSH conjugate formed/h/100 mg protein, respectively. The asterisk denotes that data are significantly different from Group I and the sharp (hash mark) denotes that data are significantly different from Group II.

TABLE 3. PROTEIN CONTENT OF RAT LENS

Parameter	Group I	Group II	Group III	Group IV
Total protein (mg/g lens)	470 \pm 27.88	414 \pm 45.96*	458 \pm 29.37#	467 \pm 36.05#
Soluble protein (mg/g lens)	392 \pm 29.91	209 \pm 46.00*	330 \pm 46.00#	340 \pm 47.01#
Soluble protein (%)	83.3	49.29	72.05	71.43

There was a significant decrease in both total and soluble protein in untreated diabetic (Group II) compared with the control (Group I) and Emblica (Group IV) and tannoids (Group III) prevented the loss of total and soluble protein in the lens. The total and soluble protein in the lens of different groups was estimated by the Lowry method and the percentage soluble protein content was derived from the estimated values. The data are mean \pm SD (n=4). The asterisk denotes that data are significantly different from Group I and the sharp (hash mark) denotes that data are significantly different from Group II.

Emblica and its tannoids prevented the alterations not only in TBARS but also in protein carbonyls despite elevated glucose levels. Simultaneously, there was an increase in specific activities of SOD, GPx, and a marginal decrease in GST in lenses of Group II animals compared with Group I which further substantiates the role of oxidative stress in cataractogenesis due to hyperglycemia (Table 2). Emblica and tannoids treatment partially prevented the altered activities of antioxidant enzymes. These data clearly demonstrate that Emblica and tannoids not only inhibited osmotic stress but also prevented hyperglycemia-induced lenticular oxidative stress probably due to the inhibition of the polyol pathway. Moreover, the tannoid rich fraction of Emblica has been shown to exert antioxidant properties in stress-induced oxidative damage model in rat brain [41,42].

TABLE 4. THE DISTRIBUTION OF CRYSTALLINS IN A SOLUBLE PROTEIN FRACTION

Peak	Group I	Group II	Group III	Group IV
α -Crystallin+HMW Peak	100	211	111	102
β -Crystallin	100	55	85	93
γ -Crystallin	100	74	112	110

Emblica (Group IV) and tannoids (Group III) treatment normalized the altered crystallin distribution in untreated diabetic (Group II). Group I: Control rats. Data are the average of three HPLC runs for the area under the curve. The relative percentage of peaks on the SEC column in untreated and treated diabetic groups was calculated by considering the area under the curve for the respective peak in Group I as 100%.

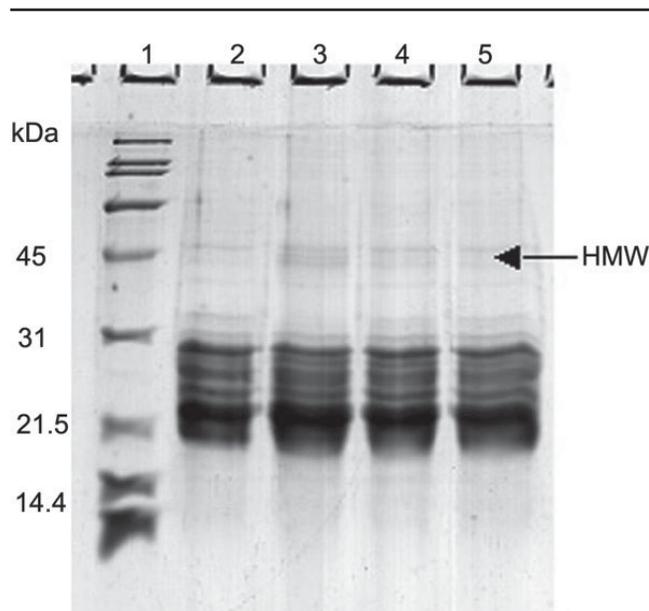


Figure 5. Subunit profile and protein cross-linking of the soluble fraction of lens. Soluble lens protein was loaded onto a 12% polyacrylamide gel under reducing conditions. Lane 1: Molecular weight markers, lane 2: Control (Group I), lane 3: Untreated diabetic (Group II), lane 4: Tannoid fed diabetic (Group III), lane 5: Emblica fed diabetic (Group IV), and HMW: High molecular weight cross links. Arrows indicate cross-linked proteins in untreated diabetic rat lens (Lane 3), which were reduced in the Emblica and tannoid treated groups.

Protein crosslinks and insolubilization: During the development of cataract, alterations in lens proteins and insolubilization have been considered to be the major change that results in lens opacification. Therefore, we analyzed the total and soluble protein content in all the groups. There was a significant decrease in both total and soluble protein in Group II compared with the control group (Table 3). This could be due to a partial leakage of proteins into the aqueous humor. Feeding of Emblica and tannoids to the diabetic rats prevented the loss of total and soluble protein in the lens. The prevention of loss in lens soluble protein in STZ-treated rats was well correlated with the delay of maturation of cataract in those groups (Figure 1). We have also studied the possible alterations or modifications in the crystallin profile due to diabetes-induced hyperglycemia by size-exclusion chromatography (SEC) as well as by SDS-PAGE. The distribution profiles of Group II lens proteins following SEC showed a decrease in β - and γ -crystallin peaks and an increase in the α -crystallin-associated high molecular weight (HMW) aggregate peak when compared to Group I (Figure 4; Table 4). The decrease in β - and γ -crystallins suggests protein degradation in diabetic cataract lens. The formation of HMW aggregates may be due to either cross-linking of degraded products or some other changes. Similar changes were also observed in our previous study [23]. By SDS-PAGE analysis, a set of protein bands corresponding to a molecular weight of about 45,000 Da was observed more prominently in Group II lenses as compared to the other groups (Figure 5). We hypothesize that these bands represent crosslinked proteins induced in the hyperglycemic lens. It is interesting to note that changes in the crystallins due to diabetes were minimized by feeding with Emblica and tannoids (Figure 4, Figure 5, and Table 4). Further studies are required to determine whether these changes might be the result of direct inhibition of polyol pathway or the result of an indirect effect involving reduced polyol pathway-mediated oxidative damage.

In this study, we demonstrated that feeding of Emblica or its constituent tannoids could delay the progression of diabetic cataract in rats. Enhanced glucose flux through the polyol pathway and increased sorbitol and fructose accumulation has been implicated in diabetes-induced cataract formation [43]. In our study, we observed increased specific activity of AR with simultaneous increased sorbitol levels in untreated diabetic rats compared with control animals. Interestingly, feeding of Emblica at a 2% level and tannoids at a 0.2% level resulted in decreased formation of sorbitol, presumably as a result of AR inhibition. Although multiple mechanisms may contribute to these effects, the antiosmotic effect of Emblica appears to be the predominant mechanism of action. Though there are reports on hypoglycemic activity of Emblica [25], in our study, we did not observe such an effect. One of the important observations of this study was that Emblica delayed the progression and maturation of cataract despite elevated levels of glucose. While the levels of Emblica and tannoids used in the study were based on a pilot study involving a small number of rats, it may be feasible to observe more pronounced effects with still higher doses. If that is achieved, delay of

cataract by treatment with Emblica/tannoids merit further attention.

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