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EVALUATION OF THE ANTI-CATARACT POTENTIAL OF TINOSPORA CORDIFOLIA & PHYLLANTHUS EMBLICA IN AN *IN VITRO* SUGAR INDUCED CATARACTERIC LENS ORGAN CULTURE MODEL

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ABSTRACT

The present study was conducted to evaluate the *in vitro* anti-cataract potential of *Tinospora cordifolia (Tc)* and *Phyllanthus emblica (Pe)*. Aldose Reductase Inhibitory (ARI) activity was evaluated using the method of Kinoshita *et al.* Only that concentration of plant extracts demonstrating maximum ARI activity was evaluated for anti-cataract and anti-oxidant potential using an *in vitro* sugar induced cataracteric lens organ culture model. Lens parameters evaluated included morphological variation, aldose reductase (AR) activity, Total Protein content and anti-oxidant enzymes *viz.,* catalase and Superoxide Dismutase. Quercetin, a pure flavonoid was used as a positive control. Both *Tc* and *Pe* demonstrated dose dependent inhibition of AR with maximum activity at 100µg/ml. Loss of lens transparency, increased AR activity, decrease in soluble and total protein content was observed in diabetic lenses as compared to normal lenses. The diabetic conditions augmented anti-oxidant enzymes indicating oxidative stress. Supplementation with *Tc* & *Pe* decreased AR levels, increased levels of total and soluble proteins significantly with a decrease in catalase and superoxide dismutase. These results were comparable to quercetin. The results suggest that these plants have the potential to arrest the progression of cataract which is mediated by their anti-oxidant activity.

KEYWORDS: **Aldose Reductase, Cataract, Catalase, Superoxide dismutase, anti-oxidant, *Phyllanthus emblica, Tinospora cordifolia***

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INTRODUCTION

Cataract is the opacity of the lens that produces painless gradual loss of vision. Cataract formation is mainly an age-related phenomenon, although socioeconomic and lifestyle factors such as nutritional deficiency, sunlight, smoking, environmental factors, lack of consumption of antioxidants may also influence its occurrence. Apart from ageing, diabetes has been considered to be one of the major risk factors of cataract.[1] Many experimental studies support the view that diabetes enhances the risk of cataract formation.[2,3]Cataractogenesis is mainly due to accumulation of excessive sorbitol in the lens fibers and consequent osmotic stress. Glucose is reduced to Sorbitol by the enzyme Aldose Reductase (AR) utilizing NADPH as a cofactor in the polyol pathway. Sorbitol does not easily cross cell membranes and accumulates in cells causing damage by disturbing osmotic homeostasis.[4] Excessive free radical generation leading to oxidative stress is another mechanism involved in the formation of cataract.[5] This oxidative stress induces the development of cataract, which leads to alteration in the activities of the antioxidant enzymes *viz.,* superoxide dismutase (SOD), catalase, glutathione peroxidase and glutathione reductase which are responsible for protection of the cells and tissues against oxidative injury. As diabetes associated phenomena such as increased AR activity and oxidative stress are interrelated, agents which inhibit AR activity represent an attractive strategy for the prevention of diabetic complications *i.e.,* cataract.

Although, a wide variety of compounds have been synthesized to inhibit AR activity and studied in experimental models, only a limited number of drugs have reached the clinical stage.[6,7] However these inhibitors have not been very successful due to their adverse effect profile.[8,9] Thus, intensive research continues to identify and test both synthetic as well as natural products for their therapeutic potential to prevent the onset as well as progression of cataract Phytotherapy has played an important role in the management of diabetes and its related complications. *Phyllanthus emblica (Pe)* and *Tinospora cordifolia (Tc)* have been scientifically validated using various *experimental* models for their use as anti-diabetic agents.[10-16] Both the plants have also been known to exhibit anti-oxidant properties. Additionally *Pe* is been reported to be beneficial to the eyes i.e., *chakshushya* in *Ayurveda* and an experimental study has identified its AR inhibitory potential.[17] Hence the present study was conducted with the objective to evaluate the ARI potential followed by evaluation of anti-cataractogenic effect of these plants using an *in vitro* model of glucose induced cataract. Further an attempt was made to explore the probable mechanism of action of these plants by evaluating the effect on catalase and SOD levels.

MATERIALS & METHODS

Materials

DL-glyceraldehyde, glucose, NADPH, quercetin and dimethylsulfoxide were obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals and solvents were of analytical grade and were obtained from local companies.

Study drugs

Standardized hydro-alcoholic extracts of *Phyllanthus emblica* (fruits) and aqueous extract of *Tinospora cordifolia* (stem) were procured from Natural Remedies, Bangalore. The authentication report and Certificate of analysis is available on file. The AR inhibitory activity of the plant extracts was evaluated over a concentration range from 10 to 100µg/ml. These concentrations were calculated from the therapeutic dose of the plants and were within the concentration range commonly used for *in vitro* studies. Quercetin the standard drug was used as a positive control. The extracts of *Pe* and *Tc* in the powdered form were dissolved in Phosphate buffer II, the buffer used in the system to achieve desired concentrations. The

concentration of the plant extracts that demonstrated the maximum ARI activity was evaluated for its anti-cataract and anti-oxidant potential in an *in vitro* sugar induced cataracteric lens organ culture model.

Rat lens

Rat lenses were used as a source of AR for AR inhibitory assay as rat lens possesses maximum AR activity. Crude AR was prepared from rat lens. Eyeballs were removed from animals (male Wistar rats, aged 8-10 weeks weighing between 150-200gms) used as Controls in other experimental projects carried out at the institutional animal house. The study was conducted following Institutional Animal Ethics Committee approval. Lenses were dissected by posterior approach and homogenized in 10 volumes of 100mM potassium phosphate buffer pH 6.2. The homogenate was centrifuged at 15,000×*g* for 30 min at 4 ˚C and the resulting supernatant was used as the source of AR.

Aldose reductase assay

The lens AR activity was measured by the method of Hayman and Kinoshita with modifications.[18] 1.4 ml of Phosphate Buffer (pH 6.2, 0.067M), 0.2 ml of NADPH (25 x 10^{-5} M) and 0.2 ml of lens supernatant was added to a sample cuvette. The mixture was then kept for incubation at 37^{o} C for 10 minutes. The enzymatic reaction was initiated by the addition of substrate DL-glyceraldehyde (5 x 10^{-4} M) and the absorbance was recorded in a spectrophotometer at 340nm for 3 minutes at 30 seconds intervals. The AR activity was expressed as △O.D/min/mg protein. For inhibition studies concentrated stocks of various plant extracts were added to assay mixture and incubated for 10 minutes before initiating the reaction by substrate DL-glyceraldehyde. The AR activity without inhibitor was considered as 100%.

Lens organ culture

Rat lenses were dissected from the eyes by posterior approach. Each isolated lens was incubated in 1ml of modified TC-199 medium in the presence of antibiotics *i.e.,* penicillin and amphotericin in a CO_2 incubator (95% air and 5% CO_2 at 37^{0}C) with 100mM glucose (supraphysiological concentration of glucose) for a period of 10 days according to the method previously described.[19] Lens incubated with 5.5mM glucose (physiological conc.) served as control. Damaged lenses were identified by determining the protein content of an aliquot of the medium after an equilibration period of 2 hrs and were discarded. Plant extracts were prepared in TC-199 and filtered before adding. Medium was changed every 48 hrs and supplemented with plant extracts and the standard drug quercetin along with glucose 100mM. All the reagents used in lens culture were filtered through 0.2 µm Millipore disc filters. Lenses were observed for development of generalized haziness or opacity, disruption and other morphological changes. After 10 days of culture the lenses were homogenized in buffer containing 25mMTris, 100mM NaCl, 0.5mM EDTA and 0.01% NaN_3, pH 8.0. The soluble fraction of homogenate (10,000×*g* for 30 min at 4 ◦C) was used for further analysis.

Estimation of Protein Content

Total protein and protein content in the supernatant of the lens homogenate was determined by the method of Lowry *et al*.[20]

Determination of catalase activity

Catalase activity was measured by the method of Aebi. [21] 0.1 ml of the supernatant was added to the cuvette containing 1.9 ml of 50 mM Phosphate buffer (pH 7.0). The reaction was initiated by the addition of 1.0 ml of freshly prepared 30 mM H_2O_2. The rate of decomposition of H_2O_2 was measured spectrophotometrically by recording the changes in absorbance at 240 nm. The activity of catalase was expressed as units/mg protein.

Determination of SOD activity[22]

0.25 ml of the supernatant was added to the reaction mixture containing 0.5 ml Tris EDTA buffer, 0.1 ml of Triton X-100 (0.01%), 0.1 ml of NBT (1mM) and the reaction was initiated by addition of Pyrogallol (2mM). The change in

absorbance was recorded spectro-photometrically at 546 nm every 30 seconds for about 4 minutes. The activity of superoxide dismutase was expressed in units/mg protein.

RESULTS

Effect on AR Inhibitory activity using rats' lens:

The hydro alcoholic extract of *Pe* and aqueous extract of *Tc were* tested over a concentration range from 10 – 125 µg/ml. *Pe* demonstrated a dose dependent response ($r = 0.871$, $p<0.05$) up to 100 µg/ml concentration at which 95.58 ± 2.61% inhibition of the enzyme activity was observed followed by complete inhibition of the enzyme activity at higher concentration (125 µg/ml). Similarly *Tc* also showed a dose dependent inhibition ($r = 0.656$; $p<0.003$) of the enzyme activity with a maximum inhibition of 83.99 ± 4.16% at 100 µg/ml followed by complete inhibition at 125 µg/ml. The inhibitory potential of both the plant extracts was comparable to quercetin (25 µg/ml) which demonstrated 83.58 ± 11.18 % of inhibition. The results are represented in Fig 1.

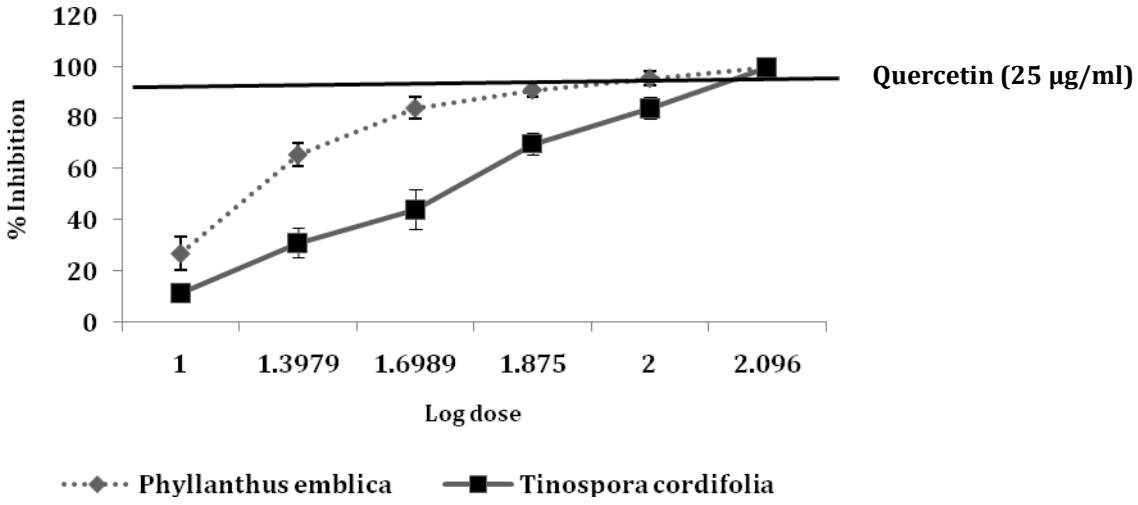


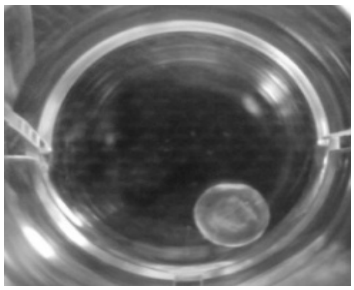
Figure 1
Inhibition of rat lens AR by hydro-alcoholic extract of Pe (⋯) and aqueous extract of Tc (—). Data represented as Mean ± SD for (n=6)

Lens organ culture

Morphological changes in lens

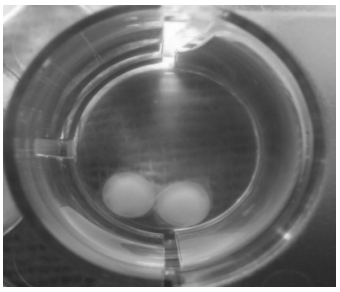
Lenses incubated with 100mM glucose (supraphysiological conc.) for 10 days exhibited an increase in opacity and became hazy representing diabetic conditions. Supplementation of *Pe* and *Tc caused* less haziness and a decrease in opacity as compared to hyperglycemic conditions. The effect was comparable to that of quercetin. The results are represented in Fig 2.

Lenses incubated with 5.5 mM glucose physiological conc.



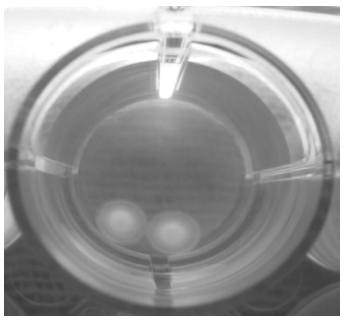
Control lenses showing clear, transparent surface

Lenses incubated with 100mM glucose supraphysiological conc.



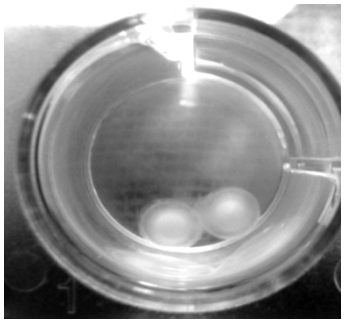
Diabetic lenses showing opacity and haziness

Lens incubated with Quercetin (10µg/ml) in presence of 100mM glucose



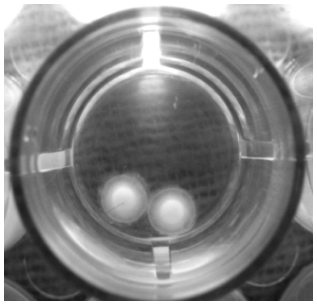
Lenses incubated with Quercetin showed increase in opacity as compared to diabetic lenses

Lens incubated with *Pe* (100µg/ml) in presence of 100mM glucose



Lenses incubated with *Pe* showed considerable difference in opacity as compared to diabetic lenses

Lens incubated with *Tc* (100µg/ml) in presence of 100mM glucose



Lenses incubated with *Tc* showed considerable difference in opacity as compared to diabetic lenses

Figure 2

Morphological Changes in Lens. Lenses incubated with 100mM glucose showed increase in opacity as compared to normal lenses. Supplementation of Pe and Tc extracts in the TC-199 medium showed a decrease in opacity

Evaluation of AR activity in Cultured lenses

As expected, a significant increase in AR activity was observed in diabetic lenses as compared to normal lenses representing *in vitro* cataract. Quercetin showed AR activity similar to that seen with normal lenses indicating its inhibitory action on AR activity. Both the plant extracts *i.e., Tc* and *Pe* at 100 µg/ml showed a minimal AR activity when compared to the AR activity exhibited by diabetic lenses and the effect was statistically significant. The results are represented in Fig 3.

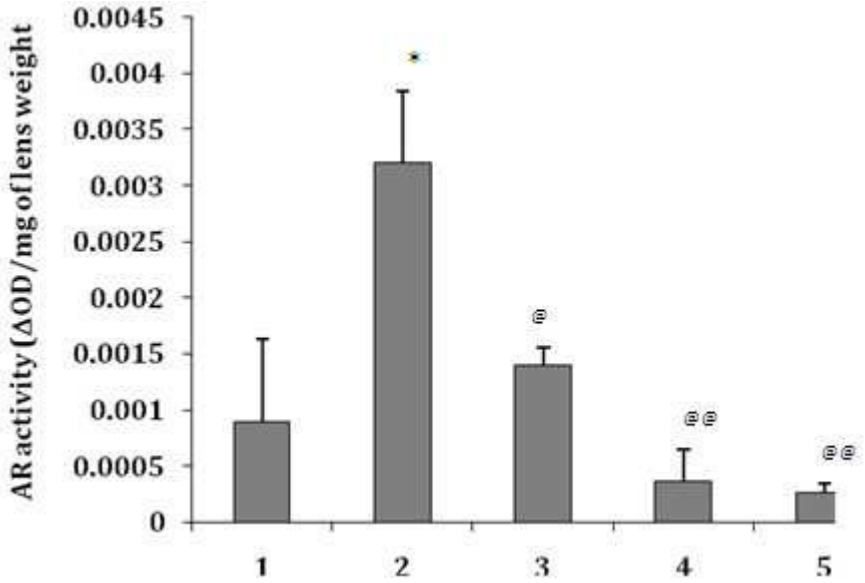


Figure 3
Effect on AR activity in cultured rat lenses

(1) AR activity in Normal lenses (5.5mM glucose concentration); (2) AR activity in Diabetic lenses (100mM glucose concentration); (3) AR activity in diabetic lenses supplemented with Quercetin (25 µg/ml); (4) AR activity in diabetic lenses supplemented with Tc (100 µg/ml) and (5) AR activity in diabetic lenses supplemented with Pe (100 µg/ml). Data represented as Mean ± SD for (n=6). *p<0.05 as compared to normal lenses; $^{@@}$p<0.01;$^{@}$p<0.05 as compared to diabetic lenses (ANOVA followed by post-hoc test)

Evaluation of protein content

A significant decrease in the total and soluble protein content in the diabetic lenses was observed as compared to the protein content of the control group. Both plant extracts showed a significant increase in the protein content as compared to the diabetic lenses and the effect was comparable to the standard group *i.e.,* quercetin treated lenses. The results are represented in Fig. 4

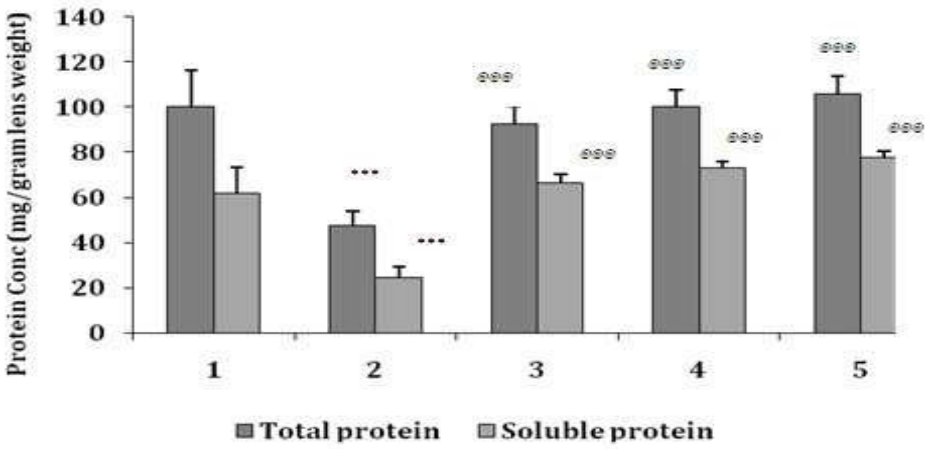


Figure 4
Protein content of cultured rat lens expressed as mg/g of lens tissue

(1) Normal lenses (5.5mM glucose concentration); (2) Diabetic lenses (100mM glucose concentration); (3) Diabetic lenses supplemented with Quercetin (25 µg/ml); (4) Diabetic lenses supplemented with Tc (100 µg/ml) and (5) Diabetic lenses supplemented with Pe (100 µg/ml). Data represented as Mean ± SD for (n=6). ***p<0.001 as compared to normal lenses; @@@p<0.001as compared to diabetic lenses control (ANOVA followed by post-hoc test)

Evaluation of Catalase activity

The diabetic lenses showed significantly increased levels of catalase as compared to normal control lenses indicating high oxidative stress. Quercetin decreased the activity of catalase indicating its anti-oxidant property. *Pe* (100 µg/ml) treated lens showed minimum catalase activity as compared to the disease control and the effect was comparable to the standard group. *Tc* (100 µg/ml) also showed a significant decrease in catalase activity when compared to normal and diabetic groups. The results are represented in Fig 5.

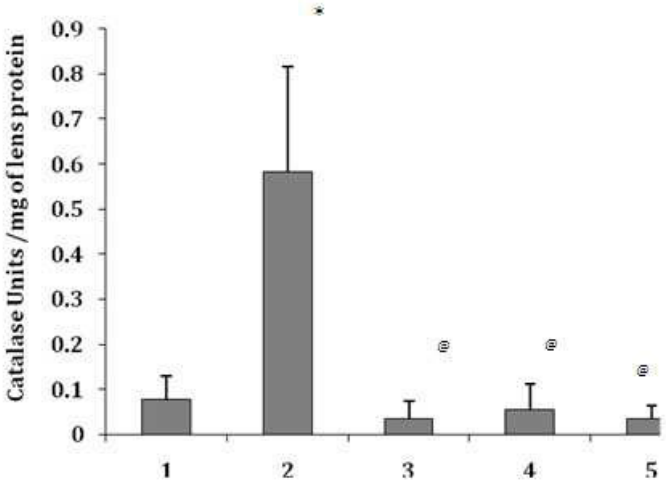


Figure 5
Catalase activity in cultured rat lens expressed as mg/g of lens tissue

(1) Normal lenses (5.5mM glucose concentration); (2) Diabetic lenses (100mM glucose concentration); (3) Diabetic lenses supplemented with Quercetin (25 µg/ml); (4) Diabetic lenses supplemented with Tc (100µg/ml) and (5) Diabetic lenses supplemented with Pe (100 µg/ml). Data represented as Mean ± SD for (n=6). *p<0.05 as compared to normal lenses; $^{@}$p<0.05as compared to diabetic lenses control (ANOVA followed by post-hoc test)

Evaluation of superoxide dismutase activity

The normal control lenses showed significantly lower levels of SOD as compared to diabetic lenses. The elevated levels of SOD indicate high oxidative stress. The SOD levels in lenses treated with plant extracts showed significant decrease in the levels as compared to diabetic lenses and the effect was comparable to that of quercetin. The results are represented in Fig 6.

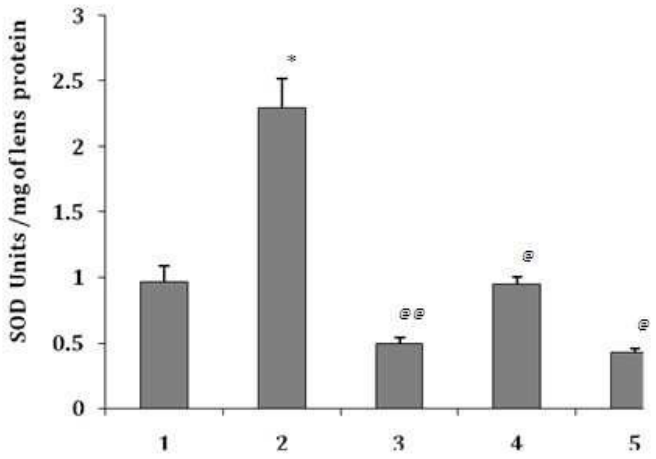


Figure 6
Superoxide Dismutase activity in cultured rat lens expressed as mg of lens protein

(1) Normal lenses (5.5mM glucose concentration); (2) Diabetic lenses (100mM glucose concentration); (3) Diabetic lenses supplemented with Quercetin (25 µg/ml); (4) Diabetic lenses supplemented with Tc (100µg/ml) and (5) Diabetic lenses supplemented with Pe (100 µg/ml). Data represented as Mean ± SD for (n=6). *p<0.05 as compared to normal lenses; $^{@}$p<0.05; $^{@@}$p<0.01as compared to diabetic lenses control (ANOVA followed by post-hoc test)

DISCUSSION

The beneficial effect of ARI in preventing or delaying the onset of cataract in experimental models provides a strong support to the hypothesis that ARI could be an effective strategy in the prevention or delay of cataract. Oxidative stress has been suggested as a common underlying mechanism of cataractogenesis, and augmentation of the antioxidant defenses of the lens has been shown to prevent or delay cataract.[23] Previous studies with experimental ARIs like GP-1447 and KIOM-79 have demonstrated a correlation between ARI and prevention of diabetic cataract progression in animal models.[24,25] Furthermore, AR deficiency has demonstrated protection against sugar-induced lens opacification in rats[26], though the clinical usefulness of this observation has been limited by early clinical indicators that these drugs may also be associated with hepatic function abnormalities, gastrointestinal symptoms such as nausea and diarrhea, and skin rash or eczema.[27] Therefore, modulation of AR activity is contemporarily regarded as an important target for ARIs designed to prevent and treat diabetic cataract and the search for exploring new ARIs continues. In our study we evaluated the ARI potential of *Pe* and *Tc* followed by evaluation of anti-cataract potential of these plants in an *in vitro* model of sugar induced cataract lens culture. Anti-oxidant activity of these plants was also studied in this model to evaluate whether the anti-cataract effect of these plants is mediated through the anti-oxidant mechanism. The inhibitory potential of the plants was calculated considering the activity of AR in the normal lenses as 0.01 i.e., 100%. The selected plants were evaluated at concentrations starting from 10µg/ml up to a limit where complete inhibition of the enzyme was observed. The concentration exhibiting maximum ARI was used further to evaluate the anti-cataract and anti-oxidant effect. It was seen that both the plant extracts, *i.e., Pe* and *Tc* demonstrated a dose dependent inhibition of AR activity, and the effect was comparable to quercetin (25 µg/ml), standard ARI used in the study.

In the glucose induced lens organ culture model, lens treated with glucose exhibited haziness and increase in opacity with a significant increase in AR activity as compared to the control lens indicating development of cataractogenic conditions. Lens supplemented with the plant extracts demonstrated a decrease in opacity with a significant decrease in AR activity substantiating the *in vitro* inhibitory potential of these plants against rat lens AR. The total and soluble protein content in lenses incubated with supraphysiological concentrations of glucose were also found to decrease when compared with control lens. It is known that during cataractogenesis large amounts of insoluble protein derived from the soluble protein gets accumulated resulting in decreased total and soluble protein.[19] However, decrease in soluble protein was significantly prevented by addition of the test drugs. The hyperglycemic conditions also augmented the catalase and SOD levels indicating oxidative stress. Further, the lens supplemented with the plant extracts demonstrated decrease in the levels of catalase and SOD levels as compared to the hyperglycemic lenses confirming the anti-oxidant potential of these plants. The AR inhibitory and anti-oxidant activity exhibited by these plants was comparable to quercetin. Hence the activity of these plants can be attributed to the presence of quercetin which is a flavonoid present in the plant extracts. A previous study has shown that flavonoids, with antioxidant properties, can prevent oxidative damage and slow experimental cataract progression.[28]

Our ARI results are also in agreement with literary data that these plants have a role in preventing the progression of cataract.[29,30] Our results also demonstrated the *chakshushya* property of *Pe* as noted in *Ayurveda.* The anti-oxidant property of the plant extracts was also confirmed in our study as seen by their effect on SOD and catalase activity. Thus we can say

that the probable mechanism by which these plants have the potential to arrest the progression of cataract is mediated through their anti-oxidant activity. Another patho-physiological mechanism behind the formation of cataract is deficient glutathione (GSH) levels, which involves keeping the lens proteins in their reduced form. Hence the effect of these plants may be studied on GSH levels to confirm the mechanism of action which is the limitation of our study. It would also be interesting to confirm these results using *in vivo* studies. Further studies using different concentrations, activity-guided isolation of active constituents from the plant extracts etc. will open up avenues for the development of these drugs.

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