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In vitro and *in vivo* evaluation of pterostilbene for the management of diabetic complications

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A B S T R A C T

Background: Aldose reductase (AR) and Advanced glycation end product (AGE) are known to play important roles in the development of diabetic complications. The inhibitors of AR and AGE would be potential agents for the prevention of diabetic complications.
Objective: The present study was aimed to evaluate the aldose reductase (AR) and advanced glycation end product (AGE) inhibitory potential of pterostilbene for its possible role in the treatment of diabetic complications such as cataract.
Materials and methods: The compound was studied for its inhibitory activity against rat lens AR (RLAR) and rat kidney AR (RKAR) in vitro along with its ability to inhibit the formation of AGEs. Anticataract activity of pterostilbene was demonstrated using sugar induced lens opacity model in isolated cattle lens. Further, the involvement of pterostilbene in galactosemia in rats was investigated by assessing the key markers in the polyol pathway and the results were compared with that of a potent AR inhibitor, fidarestat.
Results: Pterostilbene exhibited inhibitory activity against RLAR and RKAR with IC50 values of 5.49 mg/ml (21.4 mM) and 6.40 mg/ml (25.02 mM), respectively. In sugar-induced lens opacity model, pterostilbene displayed a significant protective effect by preventing opacification and formation of polyols in cattle lens. Besides, the compound exhibited in vivo inhibition of galactitol accumulation in lens and sciatic nerves of galactose fed rats.
Conclusion: The results obtained in the study underline the potential of pterostilbene as possible therapeutic agent against long-term diabetic complications.

1. Introduction

Long-term complications are the main cause of mortality and morbidity in diabetic patients [1]. Several metabolic factors contribute to the diabetic complications which include vascular complications such as nephropathy, neuropathy, retinopathy, and vascular complications like cataract [2]. Among these, cataracts are eye abnormalities causing opacification of lens leading to defects in vision and blindness. Although, the exact mechanism of diabetic cataract is unknown, several mechanisms have been proposed. The most promising mechanisms are the polyol pathway and an increase in advanced glycated end products (AGEs). The polyol pathway which is responsible for the reduction of glucose to sorbitol by the enzyme aldose reductase (AR) increases its activity during hyperglycemia. This increased concentration of sorbitol causes osmotic changes along with increased water influx and thickening of fibers and subsequent formation of cataract [3]. Another vital point is the formation of AGEs by cross-linking of lens proteins due to non-enzymatic glycosylation. These AGEs readily accumulate in the lens and cause oxidation of thiol groups, cross link formation and aggregation of the crystalline proteins producing high molecular weight insoluble proteins which are responsible for opacification of the lens [4].

Consequently, inhibitors of AR and AGEs formation have been an attractive approach for the treatment of diabetic complications including cataract formation. Although AR and the polyol pathway are promising targets for the treatment of diabetic complications, few ARIs were able to restore all aspects of polyol pathway but exhibited a poor or only a partial amelioration, with unacceptable toxicities in few studies. Despite the synthesis of various potent AR and AGE inhibitors, they could not satisfy the clinical need in the

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therapy of diabetic complications. In specific to fidarestat, though various studies exhibited significant improvement in various physiological and subjective symptoms, it was clinically not very effective in late phase of diabetic complications [5–7]. Thus, searching for improved drugs from natural sources for the management of diabetic complications may prove to be beneficial. In this sequence, in our previous report [8], we have evaluated various plants and their active constituents for their AR and AGEs inhibitory potential and in the present study, pterostilbene (PST), an active constituent of the plant *Pterocarpus marsupium*, which was found to exert significant anticataract activity [9] was selected to elucidate its possible AR and AGE inhibitory activity along with its effect on various biochemical alterations in experimental galactosemia in rats.

PST, a dimethyl ester derivative of resveratrol, with a potent antioxidant activity was found in various plants like *Pterocarpus marsupium*, *Pterocarpus santalinus*, and *Vitus vinifera*. PST is known to have diverse pharmacological benefits in the treatment of various diseases, such as cancer, dyslipidemia, diabetes, cardiovascular degeneration and pain. Further, the compound acts as a chemo-preventive, antioxidant, and anti-inflammatory agent exhibiting beneficial effect on atherosclerosis [10]. In this context, the present study was designed to elucidate the role of PST on AR and AGE inhibition and thereby its therapeutic role in diabetic complications.

2. Materials and methods

2.1. Materials

NADPH, Aminoguanidine, PST and Tri-sil HTP reagent were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Krebs ringer buffer medium and dulcitol were obtained from Hi Media Laboratories (Mumbai, India). Fidarestat was kindly donated by Symed Labs Ltd (Hyderabad India). All the other chemicals were of analytical grade.

2.2. Animals

Male Wistar rats (180–200 g) were obtained from Sanzyme Ltd (Hyderabad, India). And housed at 25 °C and relative humidity of 45–55% under a natural light: dark cycle with unrestricted access to food and water. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) and performed in accordance with the guidelines of Committee for Control and Supervision of Experimentation on Animals, Government of India on animal experimentation.

2.3. In vitro AR inhibitory activity

2.3.1. Preparation of tissue homogenates
Rats were sacrificed by cervical dislocation followed by removal of eyes and kidneys. The eye lenses were enucleated through posterior approach. Both lens and kidneys were homogenized separately with three volumes of 0.1 M sodium phosphate buffer (pH 6.2) and centrifuged at 16,000 rpm at 4 °C for 30 min and the supernatant collected was used as crude AR preparation [11]. The protein content, enzyme activity and specific activity of the enzyme preparation were determined using previously reported methods [12,13].

2.3.2. AR inhibitory assay
AR inhibitory activity assay was performed as described [14]. Briefly, pterostilbene at concentration of 1, 5 and 10 μg/ml was prepared separately in 10% DMSO. The reaction mixture consisted of 300 μl of 0.15 mM NADPH, crude enzyme preparation, 300 μl of test or standard drug solution (10% DMSO in blank) and the final volume was made up to 2.7 ml with sodium phosphate buffer. The reaction was initiated by addition of 300 μl of 10 mM DL-glyceraldehyde as substrate (double distilled water in blank) and absorbance was measured at 340 nm using double beam UV spectrophotometer (SL210, Elico, India) for 1 min at 5 s interval. Absorbance was recorded for all the concentrations in triplicate. The AR inhibitory activity of each sample was calculated using the formula:

$$\% \text{ inhibition} = \left[1 - \frac{\Delta A_{\text{Sample}} - \Delta A_{\text{Blank}}}{\Delta A_{\text{Control}} - \Delta A_{\text{Blank}}} \right] \times 100$$

where ΔA_{sample} is a decrease of absorbance per min with pterostilbene or fidarestat. ΔA_{blank} is decrease in absorbance of the blank solution, while $\Delta A_{\text{control}}$ is with 10% DMSO in place of sample solution.

2.4. Inhibition of AGEs formation in vitro

An assay was setup as described [15] to study the extent of inhibition of AGEs formation. Briefly, the reaction mixture consisted of 10 mg/ml bovine serum albumin (BSA) in 50 mM sodium phosphate buffer (pH 7.4) and 0.2 M fructose and glucose each along with 0.02% sodium benzoate. The reaction mixture was incubated in the presence of various concentrations (1, 5, 10 μg/ml) of PST or aminoguanidine (standard) at 37 °C for 7 days. The fluorescence intensity of the reaction mixture was then determined at excitation and emission wavelengths of 350 nm and 450 nm, respectively, using a fluorimeter (CL-53, Elico). The percentage inhibition of AGE formation was determined by following formula:

$$\% \text{ inhibition} = \left[\frac{\text{Florescence of control} - \text{Florescence of test}}{\text{Florescence of control}} \right] \times 100$$

2.5. Lens organ culture

Eyes of the cattle (*Bos indicus*) were obtained from a local slaughter house and the experiment was conducted on the same day. Lens were isolated from the eyes, washed with saline to be free from other tissues and incubated in sterile Krebs Ringer carbonate buffer in the presence of PST (10 μg/ml) or fidarestat (1 μg/ml) for 24 h at 37 °C with 5% CO_2 and 95% air ($n = 3$). Positive control was run with 3 mM of glucose and negative control was run without glucose in the medium [16]. After the incubation period the extent of transparency of the lens was measured by analyzing the images of the lenses using software image J v 1.47, NIH [17] followed by the estimation of polyol levels in the lens on the same day.

2.6. Estimation of polyol levels

Polyol estimation was carried out follwing the methodology as reported by Halder et al. [18]. The lenses were homogenized in 0.6 N perchloric acid followed by centrifugation at 5000 rpm for 30 min. The supernatant was separated and neutralized with 2 N KOH and again centrifuged. The supernatant obtained was reacted with 0.2 ml of 0.03 M periodic acid, 0.2 ml of stannous chloride (freshly prepared) and 2 ml of chromotropic acid (0.2%) for 30 min on a water bath to obtain a purple colored complex. The absorbance of the solution was then measured at 570 nm using double beam UV spectrophotometer (SL210, Elico, India). The concentration was measured using a parallel standard of galactitol.

2.7. Galactosemic rat model

Male Wistar albino rats weighing 180–200 g were divided into four groups ($n = 6$): Group I and Group II served as naïve and control respectively. Naive animals were treated orally with vehicle (0.5% Tween 80). Group III and group IV received fidarestat (1 mg/kg/day) and PST (10 mg/kg/day), respectively, for a period of 3 weeks by oral administration. The control and test groups were fed orally with galactose at a dose of 20 mg/kg body weight throughout the experimental period. Blood glucose was estimated for all the animals at every 7 day interval. All the animals were then sacrificed on the 21st day by decapitation followed by isolation of sciatic nerves and both eye balls. Each rat lens was then enucleated through posterior approach. The pair of lenses and nerves were thoroughly washed and cleaned of any extra tissue and stored at -20 °C until further analysis.

2.7.1. Estimation of galactitol by GLC

Right lens of each rat was homogenized separately with 1 ml of ice cold water containing methyl α-D-mannopyranoside (1 μM) as internal standard. Similarly, a part of sciatic nerve was isolated, weighed to obtain 10% homogenate. The proteins were precipitated with ethanol (70% of the final volume) and removed by centrifugation (30 min at 16,000 rpm; 4 °C). The supernatant was collected and lyophilized at -40 °C using Lyodel freeze-drier (Delvac Pumps Pvt Ltd, Chennai, India) [19]. Galactitol estimation was done by the method of [20]. Briefly, the sugar alcohols in the lyophilized samples were tri methylsilylated using Tri-sil reagent. After the addition of 1 ml of silylating agent, the reaction vial was placed in an incubating oven at 60 °C for 30 min. Analysis was performed on a GL Science GC 353; Supelco DB-1 capillary column (30 × 0.25 mm × 0.25 μM) coated with cross linked methyl silicone. Nitrogen was used as the carrier gas and column temperature was raised from 120 to 265 °C at 5 °C/min and then to 295 °C at 10 min.

2.8. AGE estimation in tissues

AGE levels in the tissues were determined by a method as previously described by Ref. [21]. Briefly, rat lens and sciatic nerves were homogenized separately in 2 ml of 0.25 M sucrose followed by centrifugation at 900 × g at 4 °C and the supernatant was separated. The pellet was re suspended in 2 ml sucrose solution and centrifuged and the supernatant obtained was mixed with the previous one. The proteins present were precipitated by adding equal volume of trichloroacetic acid (TCA). Following centrifugation at 4 °C 900 × g, the protein pellet obtained was mixed with 1 ml methanol twice to remove the lipid fraction. The insoluble protein, after washing with 10% cooled TCA was centrifuged and the residue was solubilized in 1 ml of 1 N NaOH and the protein concentration was estimated by measuring the absorbance at 280 nm against BSA standard curve. The AGEs content was then measured fluorometrically with an emission at 440 nm and excitation at 370 nm, and the results were expressed as relative fluorescence units (RFU)/mg protein.

2.9. Statistical analysis

The data were analyzed by using analysis of variance (ANOVA) followed by Dunnett's test. All the values were expressed as mean ± SEM and the criterion for statistical significance was considered to be $p < 0.05$.

3. Results

3.1. In vitro AR and AGEs inhibitory activity

Rat lens aldose reductase (RLAR) enzyme protein concentration, enzyme activity and specific activity of the lens homogenate were 1.8 mg/ml, 26.38 U/ml and 6.91 U/mg, respectively. Likewise, rat kidney aldose reductase (RKAR) crude enzyme protein concentration, enzyme activity and specific activity of the kidney homogenate were found to be 2.3 mg/ml, 8.38 U/ml and 5.16 U/mg, respectively. The IC_{50} values of PST against RLAR and RKAR were found to be 5.49 μg/ml (21.4 μM) and 6.40 μg/ml (25.02 μM), respectively, and that of the standard fidarestat IC_{50} values were found to be 0.51 μg/ml (1.8 μM) and 0.54 μg/ml (1.9 μM) for RLAR and RKAR, respectively. Similarly PST exhibited the maximum AGEs inhibitory activity at 10 μg/ml with an IC_{50} value of 6.43 μg/ml (25.14 μM) when compared to aminoguanidine which gave an IC_{50} value of 6.13 μg/ml (55.45 μM) (Table 1).

3.2. In vitro anti-cataract activity

All the lenses incubated in Krebs Ringer carbonate buffer alone remained transparent while incubation of lenses in the presence of high glucose concentration led to a decrease in transparency of the lens when compared to the negative control. However, the presence of PST or fidarestat in the high glucose medium prevented decrease in transparency (Figs. 1 and 2A). Similarly, polyol levels were significantly ($p < 0.05$) increased in lenses incubated in glucose alone when compared to the negative. The presence of PST or fidarestat in the medium significantly ($p < 0.05$) attenuated osmotic stress-induced increase in the polyol levels indicating a protective effect against the formation of cataract (Fig. 2B).

3.3. Galactosemic rat model

The body weight and blood glucose of the galactose fed rats was not significantly altered when compared to that of the normal animals. Although the blood glucose levels were regularly monitored, administration of fidarestat or PST did not affect this parameter (Table 2). However, administration of galactose led to the opacification of rat lens in control group when compared to naïve animals. Further, administration of fidarestat or PST decreased the extent of lens opacification when compared to control group (Fig. 3).

3.4. Effect on galactitol levels in tissues

Lens galactitol levels were found to be significantly ($p < 0.05$) higher in control rats when compared to naïve animals. Fidarestat

Table 1
Effect of pterostilbene on RLAR, RKAR and AGEs inhibition.

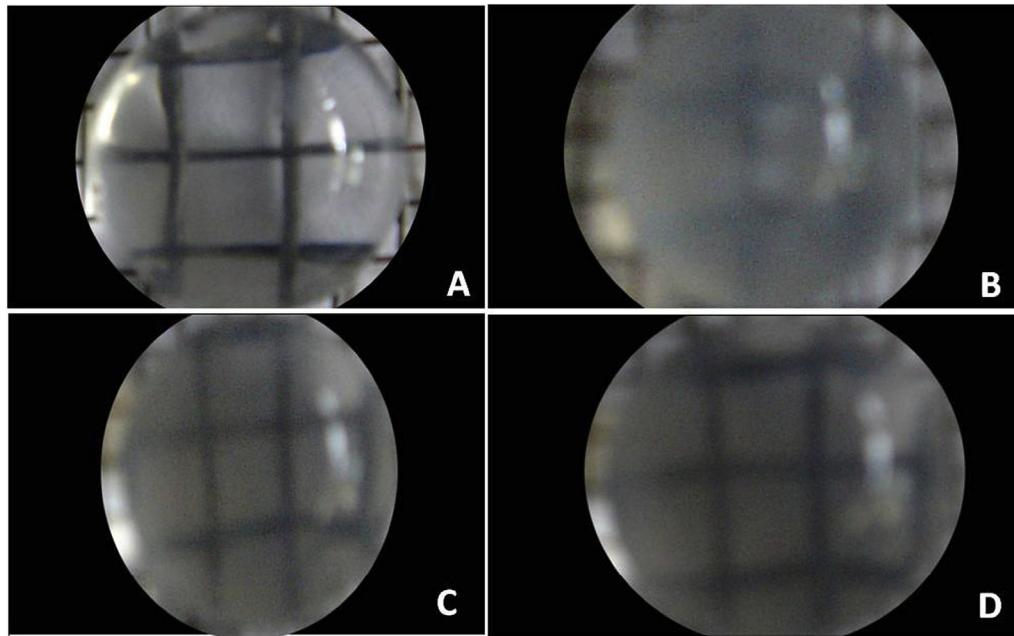| Activity	Concentration (μg/ml)	% inhibition	IC_{50} (μg/ml)	IC_{50} (μM)
RLAR	1	7.45 ± 0.96	5.49	21.4
	5	38.32 ± 1.48		
	10	88.92 ± 2.40		
RKAR	1	8.63 ± 1.04	6.4	25.02
	5	42.48 ± 1.71		
	10	79.06 ± 2.47		
AGEs	1	26.19 ± 0.93	6.43	25.14
	5	42.85 ± 1.96		
	10	73.80 ± 1.82		

Fig. 1. Morphological representation of lens and their transparencies in sugar induced opacity model in cattle lens. A: Positive control, B: Negative control, C: Fidarestat (1 μg/ml), D: Pterostilbene (10 μg/ml).
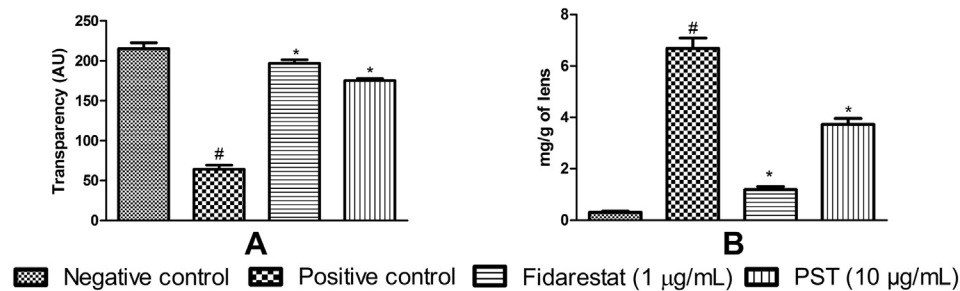

Fig. 2. Effect of pterostilbene on alterations in transparency (A) and polyol levels (B) in sugar induced opacity model in cattle lens. Data were analyzed by one way ANOVA followed by Dunnett's test. ($n = 3$); #$p < 0.05$ as compared to negative control, *$p < 0.05$, as compared to positive control. PST: Pterostilbene.

Table 2
Effect of pterostilbene on blood glucose levels (mg/dL) in galactosemic rat model.

Day	Naïve	Control	Fidarestat (1 mg/kg)	PST (10 mg/kg)
0	121.42 ± 6.26	118.47 ± 5.96	124.31 ± 6.32	122.35 ± 8.35
7	122.55 ± 5.36	124.36 ± 5.27	120.42 ± 8.37	119.33 ± 7.24
14	118.43 ± 7.33	121.17 ± 4.27	125.52 ± 5.36	124.46 ± 6.23
21	120.46 ± 5.91	125.37 ± 6.18	129.63 ± 5.07	122.34 ± 7.11

Data were analyzed by one way ANOVA followed by Dunnet's test. Values are expressed as mean ± SEM. ($n = 6$).

and PST significantly ($p < 0.05$) inhibited the accumulation of galactitol in the lens when compared to control. Similarly, galactitol accumulation in the sciatic nerve was found to be significantly higher in galactose treated control group when compared to the naïve animals. Simultaneous administration of fidarestat or PST along with galactose significantly ($p < 0.05$) prevented galactitol accumulation in sciatic nerve (Fig. 4).

3.5. Effect on AGEs formation

Induction of galactosemia in rats led to a significant increase in AGEs levels in lens and sciatic nerves when compared to naïve animals. However, administration of PST (10 mg/kg) significantly ($p < 0.05$) reduced the AGEs levels when compared to control group. Similarly, administration of fidarestat (1 mg/kg) led to a significant change in AGEs levels both in lens and sciatic nerves when compared to control group (Fig. 5).

4. Discussion

Among the various mechanisms that are involved in the pathogenesis of diabetic complications, there is an increasing interest in the effects induced by polyol pathway along with AGEs formation over various biochemical and structural changes leading to alteration in physiological functions. Several investigators now believe that a correlation exists between these alterations and appearance of diabetic complications [21,22]. The present study demonstrates that PST exhibits ARI activity against RLAR and RKAR along with AGEs inhibition *in vitro*, suggesting its potential in the treatment of diabetic complications. The results of this study prompted to further evaluate its effect on cataract formation *in vitro* and galactosemic rat model *in vivo*.

Several studies including the transgenic animal models over expressing AR confirm the role of the enzyme in the development
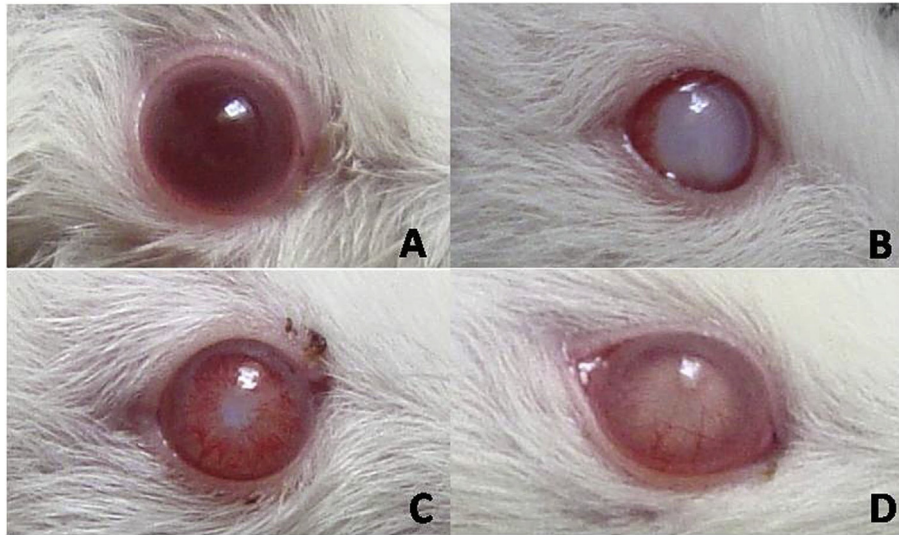
Fig. 3. Cataract formation in galactosemic rat model. A: Naive, B: Control, C: Fidarestat (1 mg/kg), D: Pterostilbene (10 mg/kg).
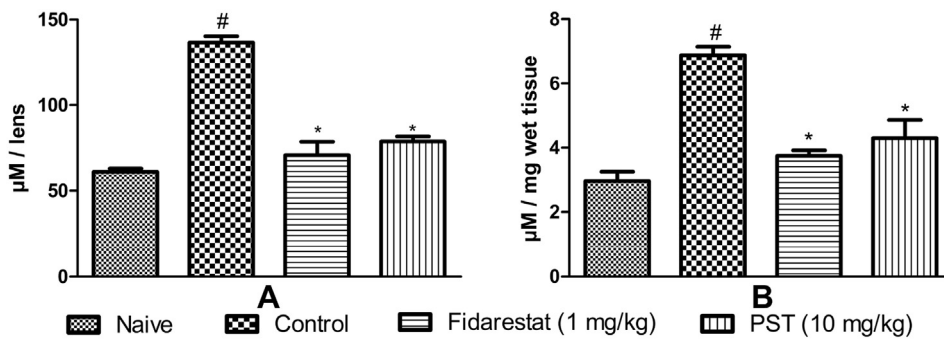

Fig. 4. Effect of pterostilbene on galactitol levels of rat lens (A) and sciatic nerve (B) in galactosemic rat model. Data were analyzed by one way ANOVA followed by Dunnett's test. ($n = 6$); #$p < 0.05$ as compared to naïve animals, *$p < 0.05$, as compared to control group. PST: Pterostilbene.
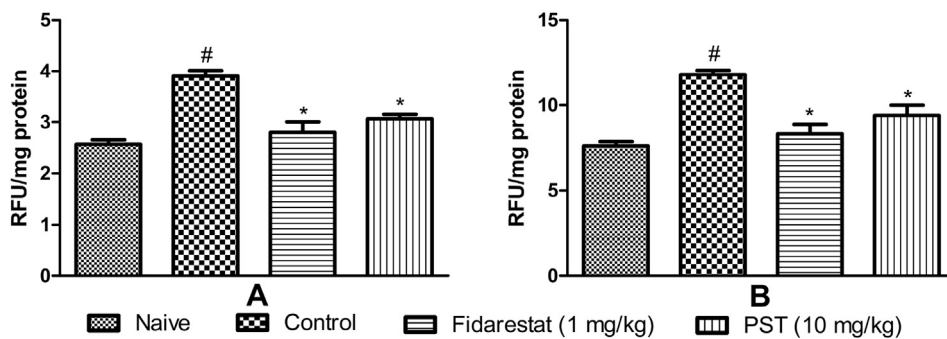

Fig. 5. Effect of pterostilbene on AGEs formation in rat lens (A) and sciatic nerve (B) in galactosemic rat model. Data were analyzed by one way ANOVA followed by Dunnett's test. ($n = 6$); #$p < 0.05$ as compared to naïve animals, *$p < 0.05$, as compared to control group. PST: Pterostilbene.

of diabetic cataracts [23]. Sugar cataracts, which occur in both hyperglycemia and galactosemia, are linked to the accumulation of excess amount of polyols. The elevated level of these impermeable polyols leads to an increase in osmotic stress thereby causing excessive hydration with loss of membrane integrity. Besides this, a second mechanism is based on the cross-linking of lens protein by nonenzymatic glycosylation leading to the formation of more stable proteins which readily accumulate a very high molecular weight insoluble AGEs responsible for the opacification of lens [24]. In

accordance with the previous reports [25], *in vitro* anticataract activity is generally evaluated using isolated cattle lens. In the present study, PST was evaluated for its ARI activity mediated anticataract potential against high glucose induced opacity and biochemical changes in bovine lenses maintained in organ culture medium. In consistent with the above theory, incubation of lenses in the high glucose medium led to the opacity of the lens when compared to that incubated in low glucose medium. The presence of PST or fidarestat in the medium prevented the induction of

opacity which can be attributed to the inhibition of lens AR by the compounds. This can be supported by the fact that the accumulation of polyols was significantly prevented by the presence of PST or fidarestat in the high glucose medium.

Galactosemia, similar to that of hyperglycemia, activates the polyol pathway with accumulation of galactitol and various other polyols which has a compensatory effect on other organic osmolites [26] and since these osmolites, especially myoionositol depletion has been invoked as an important mechanism in diabetic complications like neuropathy and cataract, it has been understood that these may serve as useful models for the study of consequent polyol-pathway related metabolic abnormalities occurring in diabetic complications and to test their responsiveness to treatment with ARIs [27]. However, since the present compound, PST is a well known antihyperglycemic agent [28], galactosemic model has been chosen instead of streptozotocin induced diabetes model to establish its *in vivo* ARI activity. The galactosemic rat model is frequently used model for aldose reductase-catalyzed disposal of excess glucose and the consequential biochemical changes in the tissues. The galactosemic model is particularly appropriate for evaluating aldose reductase inhibitors *in vivo* and is widely used model to assess the role of polyol pathway for the reason that galactose is a better substrate for aldose reductase than glucose in rats. Further, the ingested galactose is readily reduced to galactitol which is a poor substrate for sorbitol dehydrogenase leading to the accumulation galactitol in various tissues. In contrast, the glucose levels remain unaltered in the galactosemic rats when compared to the normal rats [29]. In the present study, feeding of rats with galactose did not produce any significant difference in the body weight or blood glucose throughout the experimental period. The results obtained in the present model of galactosemia were in accordance with the earlier reports where induction of galactosemia does not produce significant modulation of glucose levels in the blood [30]. Further, the glucose recording in the present study provided supporting evidence that the galactosemic model even though does not modulate the glucose levels in the body, it activates polyol pathway leading to accumulation of galactitol and thereby various physiological changes similar to that of diabetic complications. We have established the previously reported data [31] indicating the increase in the galactitol levels in the lens and sciatic nerves of galactose fed rats. Further, treatment with PST was accompanied by prevention of galactitol accumulation signifying its ARI activity *in vivo*.

It is well known that AGEs which have been implicated in the structural and functional alterations of various tissues by means of interactions with cells or matrix leading various complications. The data obtained in the present study is in accordance with the previous reports where induction of galactosemia in rats leads to the increase in AGEs in the tissues which can be prevented by administration of ARI [32]. Similar effect of decreased AGEs was found by administration of PST which further provides an evidence for its beneficial effect against diabetic complications.

5. Conclusion

To the best of our review of scientific literature, this is the first report on AR and AGE inhibition by pterostilbene. In conclusion, the data from the present study along with the previous reports on its significant antihyperlgycemic, antioxidant activity and decreased expression of AR [33], strongly argues the role of PST in the therapy of diabetes associated complications. Needless to say that further experiments have to be carried out in order to evaluate a precise therapeutic value of the compound.

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