

Esculetin, a Coumarin Derivative, Inhibits Aldose Reductase Activity *in vitro* and Cataractogenesis in Galactose-Fed Rats

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Abstract

Naturally occurring coumarin compounds have received substantial attention due to their pharmaceutical effects. Esculetin is a coumarin derivative and a polyphenol compound that is used in a variety of therapeutic and pharmacological strategies. However, its effect on aldose reductase activity remains poorly understood. In this study, the potential beneficial effects of esculetin on lenticular aldose reductase were investigated in galactose-fed (GAL) rats, an animal model of sugar cataracts. Cataracts were induced in Sprague-Dawley (SD) rats via a 50% galactose diet for 2 weeks, and groups of GAL rats were orally treated with esculetin (10 or 50 mg/kg body weight). In vehicle-treated GAL rats, lens opacification was observed, and swelling and membrane rupture of the lens fiber cells were increased. Additionally, aldose reductase was highly expressed in the lens epithelium and superficial cortical fibers during cataract development in the GAL rats. Esculetin reduced rat lens aldose reductase (RLAR) activity *in vitro*, and esculetin treatment significantly inhibited lens opacity, as well as morphological alterations, such as swelling, vacuolation and liquefaction of lens fibers, via the inhibition of aldose reductase in the GAL rats. These results indicate that esculetin is a useful treatment for galactose-induced cataracts.

Key Words: Aldose reductase, Esculetin, Sugar cataract, Galactose, Lens fibers

INTRODUCTION

A cataract is a clouding of the eye's lens that causes a progressive, painless loss of vision. Although this ocular disorder is the leading cause of blindness worldwide, the precise mechanism responsible for this phenomenon is unknown. The enzyme aldose reductase catalyzes the reduction of glucose to sorbitol through the polyol pathway, which is a process that is linked to cataract development in diabetes. Because sorbitol does not readily diffuse across cell membranes and because it demonstrates slow conversion to fructose, sorbitol accumulation under hyperglycemic conditions induces osmotic stress within the cell, leading to lens fiber cell swelling and eventually to membrane rupture (Kinoshita, 1974). Because aldose reductase is primarily localized to the lens epithelium, an increase in osmotic stress via sorbitol accumulation occurs in these cells first (Murata *et al.*, 2001).

The inhibition of aldose reductase has been proposed as a therapeutic approach to ameliorate or prevent long-term diabetic complications, such as sugar cataracts. Coumarins have a ring structure that is similar to that in flavonoids, and

an aldose reductase inhibitory activity of coumarins has also been reported (Wang *et al.*, 2008). Coumarin, a natural substance found in various plants, is an organic compound in the aroma benzopyrone chemical class and is a colorless crystalline substance in a standard state. Okuda *et al.* have reported the inhibitory effects of coumarins, flavonoids, and flavones on rat lens aldose reductase (RLAR) (Okuda *et al.*, 1982), and Brubaker *et al.* have investigated a series of synthesized coumarins using RLAR (Brubaker *et al.*, 1986). We have previously confirmed that scopoletin, a coumarin derivative, potentially prevents cataractogenesis in an *ex vivo* model, in addition to sugar cataracts in galactose-fed rats, by inhibiting aldose reductase activity (Lee *et al.*, 2010; Kim *et al.*, 2013). The structure of esculetin is very similar to that of scopoletin. Esculetin is an abundant compound found in many medicinal plants, such as *Artemisia capillaries*, *Artemisia scoparia*, *Citrus limonia* and *Ceratostigma willmottianum* (Subramaniam and Ellis, 2011). It has been reported to have various biochemical and biological effects, including anti-proliferative and anti-oxidant activities (Egan *et al.*, 1990; Lin *et al.*, 2000; Wang *et al.*, 2002; Kok *et al.*, 2009). Esculetin has also recently been

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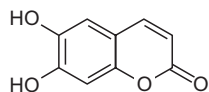


Fig. 1. Chemical structure of esculetin.

reported to have a protective effect in diabetic nephropathy by attenuating hyperglycemia-mediated oxidative stress (Prabakaran and Ashokkumar, 2013). Further, it exhibits inhibitory activity on RLAR *in vitro* (Jung *et al.*, 2011). In this study, we demonstrated the effect of esculetin on cataract development in galactose-fed (GAL) rats.

MATERIALS AND METHODS

Determination of RLAR inhibitory activity *in vitro*

Aldose reductase activity was assayed spectrofluorometrically by measuring the decrease in the absorption of NADPH according to a previously described method (Kim *et al.*, 2008). Rat lenses were removed from Sprague-Dawley rats weighting 240-260 g and preserved by freezing until use. Then, they were mixed with sodium phosphate buffer, the homogenate was centrifuged at 10,000 rpm (4°C, 20 min), and the supernatant was used as an enzyme source. Esculetin was purchased from Sigma (St. Louis, MO, USA). Briefly, the incubation mixture contained 100 mmol/l Li₂SO₄, 0.04 mmol/l DL-glyceraldehyde, 0.03 mmol/l NADPH, 135 mmol/l sodium/potassium-phosphate buffer (pH 7.0) and 100 µl of an enzyme preparation, RLAR homogenate with or without 50 µl esculetin or positive inhibitor, for a total volume of 1.0 ml. RLAR activity was determined by measuring the decrease in NADPH at excitation (360 nm) and emission (460 nm) wavelengths over 5 min using a spectrofluorophotometer (Synergy™ HT, Bio-Tek, VT, USA) at room temperature (RT). The concentration of esculetin leading to a 50% inhibition of aldose reductase activity (IC₅₀) was expressed as the mean ± SEM of triplicate experiments.

Animals and experimental design

Male Sprague-Dawley rats weighing 180-200 g were randomly divided into 4 groups of 6 animals each. The normal control group (group I) received a normal diet, group II received a 50% galactose diet (GAL), and groups III and IV were treated orally with two concentrations of esculetin (10 and 50 mg/kg body weight) with GAL once per day for 2 weeks. All other groups were also treated once per day for 2 weeks. All animal procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the Korea Institute of Oriental Medicine Institutional Animal Care and Use Committee.

Analysis of cataract formation

After two weeks of treatment, the rat eyes were excised under deep anesthesia via intraperitoneal injection of 10 mg/kg zolazepam (Zoletil, Virbac, Carros, France) mixed with 10 mg/kg xylazine hydrochloride (Rumpun, Bayer, Frankfurt, Germany). The lenses were removed from the eyeballs under an optical microscope, and the wet weights were calculated. The lenses were then transferred onto 24-well plates and photographed under an optical microscope with a CCD camera. The

opaque areas of the lenses were analyzed using an imaging program (ImageJ, NIH, USA). The data are expressed as the percentage of the opaque area relative to the entire lens area.

Analysis of lens fiber degeneration

The isolated lenses were then fixed in 2% paraformaldehyde and 1% glutaraldehyde for 48 h, embedded in paraffin, and sectioned (6 µm). To assess lens fiber degeneration, the sections were next reacted with rhodamine-conjugated wheat germ agglutinin (Vector Laboratories, CA, USA) for 90 min. Images were captured using an Olympus BX51 microscope and DP71 digital camera (Olympus, Tokyo, Japan). The severity of lens fiber degeneration was evaluated using the following classifications: grade 0, no change in the lens fiber morphology; grade I, disorganization observed at a part of the lens fiber membrane; grade II, disorganization observed at all parts of the lens fiber membrane; grade III, morphological changes, such as swelling, of the lens fiber membrane; and grade IV, morphological changes, such as vacuolation and liquefaction, of the lens fiber membrane.

Immunohistochemical staining

For aldose reductase immunohistochemical staining, the antigen was retrieved by heating the slides in a pressure cooker in citric buffer (pH 6.0) for 10 min. Endogenous peroxidase activity was inactivated by incubation in 0.3% (v/v) hydrogen peroxidase solution in methanol for 10 min at RT. The sections were incubated at RT with goat anti-aldose reductase antibody (1:500 in 1% BSA-PBS, 2 h; Santa Cruz, CA, USA). Following three consecutive washes in PBS for 5 min each, the sections were incubated at RT for 60 min with HRP-conjugated anti-goat IgG. For signal detection, the reaction was visualized using 3,3'-diaminobenzidine tetrahydrochloride reagent (Vector Laboratories, CA, USA) for several seconds. Photographs were taken using an Olympus BX51 microscope and DP71 digital camera (Olympus, Tokyo, Japan). For morphometric analysis, the positive signal intensities per unit area (0.32 mm²) in a total of 10 randomly selected fields were determined using Image J software (NIH, Bethesda, MD, USA).

Statistical analysis

The values are presented as the mean ± SEM. The results were compared using a one-way analysis of variance (ANOVA) followed by Tukey's test. A value of *p*<0.01 was considered significant.

RESULTS

Esculetin inhibits RLAR activity *in vitro*

Esculetin was tested for its inhibitory activity on RLAR activity *in vitro*. As shown in Table 1, esculetin dose-dependently inhibited the aldose reductase activity (IC₅₀=18.11 ± 0.95 µM). The inhibitory activity of esculetin was greater than that of 3,3-tetramethyleneglutaric acid (TMG), a well-known aldose reductase inhibitor (IC₅₀=28.81 ± 1.52 µM).

Lens opacification analysis

At the end of the two-week study, no lenses displayed cataract formation in the normal rats. The lenses of the vehicle-treated GAL rats became severely opaque. However, the esculetin-treated rats exhibited the dose-dependent inhibition of

lens opacification (Fig. 2A). Quantitative analysis revealed that the mean opaque region of the lenses from the vehicle-treated GAL rats was significantly enhanced by eight-fold compared with the normal rats. Galactose-induced lens opacification was inhibited by esculetin administration in a concentration-dependent manner (Fig. 2B, $p < 0.01$). This observation indicates that esculetin prevents the onset of galactose-induced cataracts.

Lens wet weights

The amount of lens swelling was measured by determining the lens wet weights of the rats (Patterson and Bunting, 1966). As shown in Fig. 3, the lens weights of the GAL rats increased

during lens opacification. However, esculetin administration significantly decreased the lens weights compared with those of the vehicle-treated GAL rats.

Degeneration of lens fiber cells

Labeling of the lens membranes demonstrated that normal lenses consistently showed complete crystal packing of fiber cells. In vehicle-treated GAL rats, swelling, multiple membrane rupture and large areas of liquefaction of lens fiber cells were detected (40% were in grade III and 60% were in grade IV, Fig. 4). However, the highest dose of esculetin inhibited these histological changes of lens fibers (70% were in grade I and 30% were in grade II, Fig. 4).

Table 1. Inhibitory effect of esculetin on RLAR activity *in vitro*

Sample	Concentration (μM)	Inhibition (%)	IC ₅₀ (μM)
Esculetin	5.6	13.89 \pm 5.01	18.11 \pm 0.95
	14.0	38.89 \pm 6.36	
	28.1	78.70 \pm 6.26	
TMG ^a	20	31.42 \pm 5.71	28.81 \pm 1.52
	30	56.42 \pm 9.60	
	40	69.69 \pm 8.15	

Inhibitory activity is expressed as the mean \pm SEM of triplicate samples. IC₅₀ values were calculated using GraphPad Prism[®] software and plotted. ^aTMG (3,3-Tetramethyleneglutaric acid) was used as a positive control.

Expression of aldose reductase protein in the lens

In the vehicle-treated GAL rats, an increase in immunoreactive staining for aldose reductase was observed in the cytoplasm of the lens epithelium, extending into the deeper cortical fibers. However, esculetin administration dose-dependently reduced expression of aldose reductase protein in the lens epithelium and inhibited the extension of aldose reductase beneath the epithelial region (Fig. 5A). Quantitative analysis revealed that the intensity of aldose reductase immunolabeling was significantly increased by 24-fold in the vehicle-treated GAL rats compared with the normal rats, and it was suppressed by esculetin administration (Fig. 5B, $p < 0.001$).

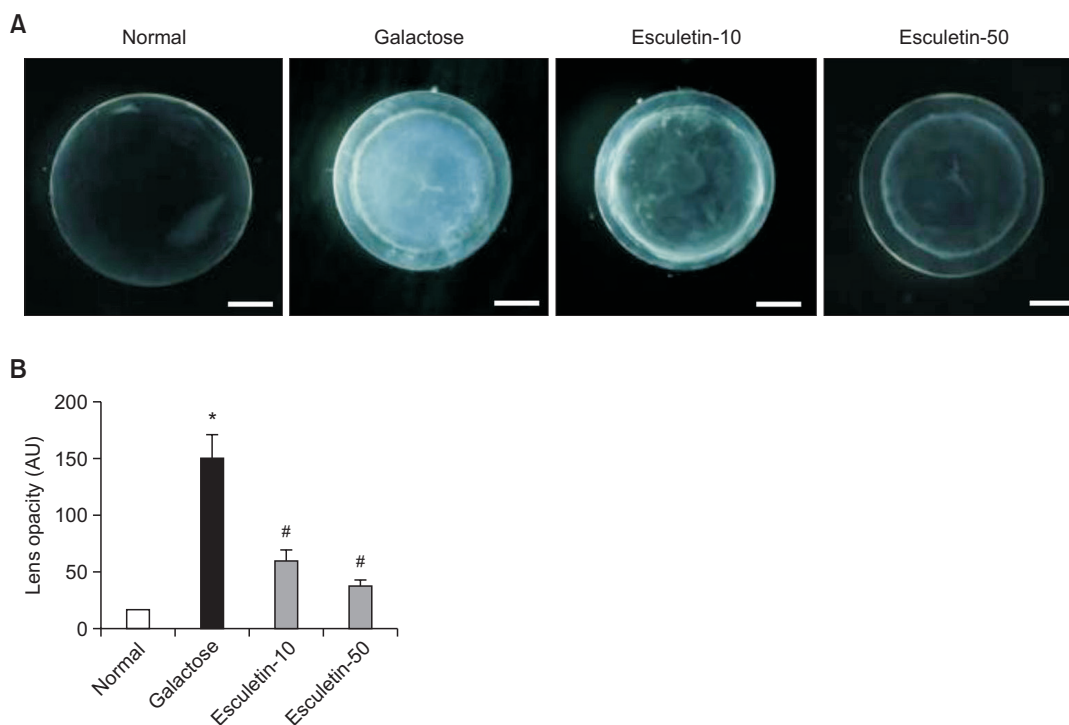


Fig. 2. Opacification of the lens. (A) Representative images of the lenses in each group. (B) Analysis of lens opacities. All opacities were analyzed in each lens of the normal rats (NOR) and vehicle-treated galactose-fed (GAL) rats. The GAL rats were treated with esculetin at doses of 10 mg/kg (Esculetin-10) and 50 mg/kg (Esculetin-50). All data are expressed as the mean \pm SE, $n = 6$. * $p < 0.01$ vs. normal rats, # $p < 0.01$ vs. GAL rats.

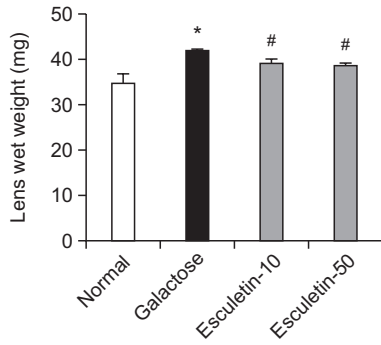


Fig. 3. Wet weights of the lenses. Normal rats (NOR), vehicle-treated galactose-fed (GAL) rats, and GAL rats treated with esculetin at doses of 10 mg/kg (Esculetin-10) and 50 mg/kg (Esculetin-50) were examined. All data are expressed as the mean ± SE, n=8. **p*<0.01 vs. normal rats, #*p*<0.01 vs. GAL rats.

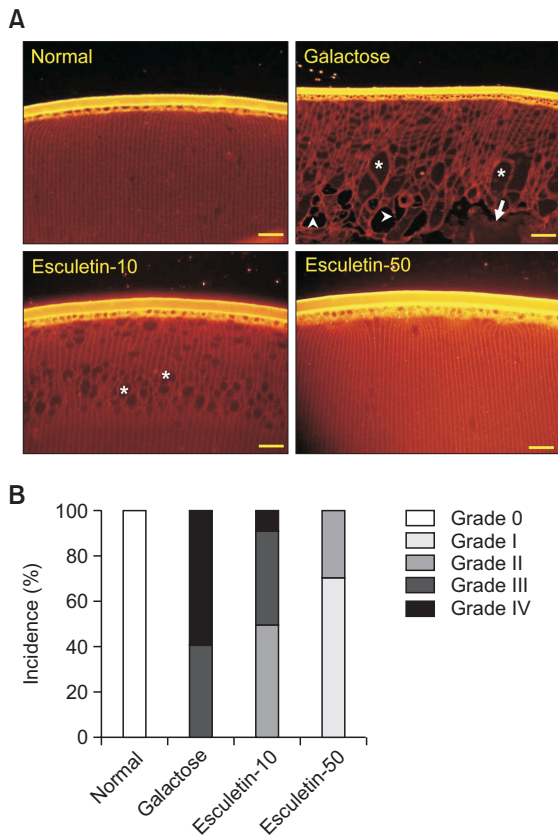


Fig. 4. Degeneration of the lens fiber. (A) Rhodamine-conjugated wheat germ agglutinin staining. Lens sections from a normal rat (NOR) and vehicle-treated galactose-fed (GAL) rat. The GAL rat samples were treated with esculetin at doses of 10 mg/kg (Esculetin-10) and 50 mg/kg (Esculetin-50). Fiber cell liquefaction (arrow), swelling (asterisk) and membrane rupture (arrowhead) were observed in the galactosemic cataractous lenses. The Scale bar indicates 20 μm. (B) Lens fiber degeneration grading. Lens fiber degeneration was assessed on a scale of a scale of 0-IV.

DISCUSSION

Cataracts represent the most common ocular disease that

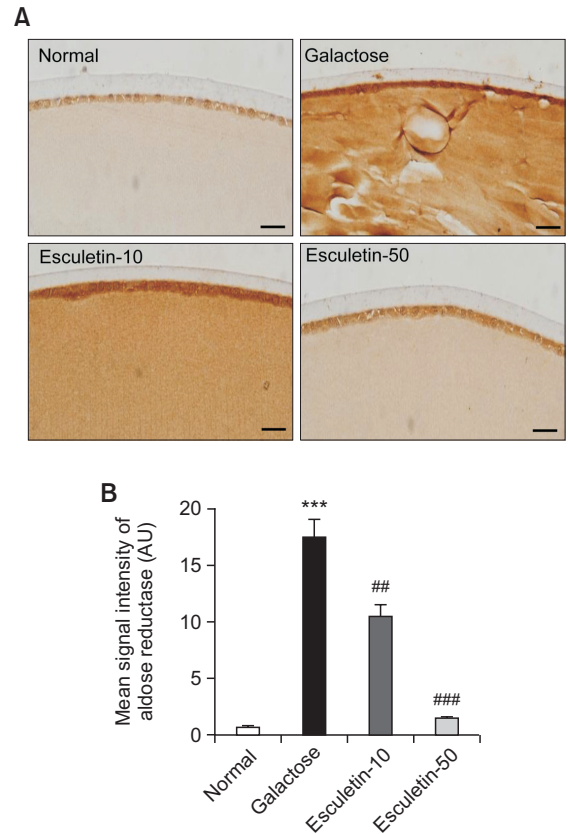


Fig. 5. Expression of aldose reductase protein. (A) Immunohistochemical staining of aldose reductase. Representative immunostaining of aldose reductase in lenses from a normal rat (NOR) and vehicle-treated galactose-fed (GAL) rat. The GAL rats were treated with esculetin at doses of 10 mg/kg (Esculetin-10) and 50 mg/kg (Esculetin-50). Strong immunoreactivity of aldose reductase was observed in the cytoplasm of lens epithelial cells and lens cortical fibers. The Scale bar indicates 20 μm. (B) Quantitative analysis of aldose reductase signal intensity. All data are expressed as the mean ± SE, n=6. ****p*<0.001 vs. normal rats. ###*p*<0.001, ##*p*<0.05 vs. GAL rats.

causes visual impairment; however, pharmacological interventions to delay or prevent the development of intraocular lens opacity remain in the experimental stage. Numerous factors are associated with the progression of this eye disease, but the exact mechanism of cataractogenesis remains unclear. Current studies are attempting to describe the mechanism of cataract formation using various animal cataract models. Galactose-induced cataract models are widely used, and the initiation factors for galactosemic cataracts in young rats can reasonably be assumed to be the same as those involved in the development of human galactosemic cataracts (Kinoshita, 1974). In this study, we evaluated the anti-cataract effects of esculetin in GAL rats using histological and morphological approaches. Treatment with esculetin had a positive effect by reducing the extent and preventing the progression of cataract formation.

Unakar and Tsui described morphological changes in the lenses of GAL rats (Unakar and Tsui, 1983), such as swelling, vacuolation and liquefaction of lens fibers, beginning in the equatorial region and progressing toward the anterior and

posterior cortical regions. As a result, the epithelial cells and cortical fibers of the lenses exhibited substantial damage, including cell membrane disruption. Sugar cataracts are osmotically induced by the intracellular accumulation of sorbitol produced from glucose, as catalyzed by aldose reductase. Recent studies have focused on the crucial role of the enzyme aldose reductase as the initiating factor in galactosemic cataract formation. Aldose reductase catalyzes the reduction of glucose to sorbitol in the first step of the polyol pathway, a process involved in galactosemic cataract development. Because sorbitol cannot cross cell membranes, it accumulates in lens epithelial cells and exerts osmotic pressure on cells by drawing in water. The increased sorbitol accumulation generates a hyperosmotic effect that induces fluid infusion to offset the osmotic gradient (Kinoshita, 1974; Kinoshita *et al.*, 1981). In animal studies, sorbitol accumulation within the lens has been shown to lead to progressive liquefaction of the lens cortex and collapse of the lens capsule, eventually resulting in lens opacity (Kinoshita, 1965, 1974). These observations have led to the hyperosmotic theory of sugar cataractogenesis, which states that the intracellular increase in fluid in response to the aldose reductase-mediated accumulation of sorbitol results in lens fiber swelling associated with histological and morphological changes, which ultimately lead to lens opacity (Kador and Kinoshita, 1984; Kinoshita, 1974; Kinoshita *et al.*, 1981). In this study, esculetin prevented the progression of lens opacity in GAL rats and also prevented galactose-induced alterations in lens morphology, such as lens fiber swelling and membrane rupture.

The inhibition of aldose reductase has been proposed to ameliorate or prevent sugar cataract formation in experimental animals (Dvornik *et al.*, 1973; Veeresham *et al.*, 2014). Although several synthetic inhibitors of aldose reductase (including epalrestat, sorbinil and tolrestat) have shown powerful effects, their uses are limited. In particular, these inhibitors have been withdrawn from clinical trials due to relatively poor permeation, low efficacy and safety issues (Kawanishi *et al.*, 2003; Manzanaro *et al.*, 2006; Peyroux and Sternberg, 2006). Among these aldose reductase inhibitors, only epalrestat is available in the Japanese market for the treatment of diabetic neuropathy. Thus, there is an urgent need for new aldose reductase inhibitors (de la Fuente and Manzanaro, 2003). Interestingly, several herbal extracts and nutritional supplements (such as naturaceuticals) have shown anti-cataract activities. For example, an extensive literature survey has shown that aldose reductase inhibition with natural resources can delay or prevent cataract formation (Crabbe and Goode, 1998; de la Fuente and Manzanaro, 2003; Kim *et al.*, 2010). Indeed, many natural compounds are known to exert potent inhibitory effects on aldose reductase *in vitro* (Tomlinson *et al.*, 1994). Compounds derived from plants with aldose reductase inhibitory activity can be classified into specific chemical groups, such as alkaloids, phenolics, flavonoids and coumarins. Among these compounds, esculetin, a coumarin derivative, has also been shown to have potent aldose reductase inhibitory activity (Jung *et al.*, 2011). In our *in vitro* assay, esculetin was found to exhibit strong inhibitory activity against RLAR, with a mean IC₅₀ value of 18.11 ± 0.95 μM, whereas Jung *et al.* have demonstrated that esculetin isolated from *Artemisia montana* has an IC₅₀ value of 172.48 ± 9.01 μM. The differences in strength of the RLAR inhibitory activity of esculetin might be attributed to differences in purity between synthetic and plant-derived

compounds and differences in the experimental methods used, such as variations in the amounts of crude aldose reductase in rat lens homogenates and incubation times. Kato *et al.* have demonstrated that esculetin treatment (50 mg/kg body weight) completely inhibits hyperglycemia after 15 and 30 min using a sucrose loading test and that it significantly suppresses the accumulation of sorbitol in erythrocytes (Kato *et al.*, 2008). However, the anti-cataractogenesis activities of esculetin in animal studies remain unclear. In our analysis of *in vitro* RLAR activity, esculetin also showed excellent inhibitory activity against aldose reductase (IC₅₀ value of 18.11 ± 0.95 μM), and this RLAR activity corresponded with anti-cataractogenic activity in the GAL rats. Our results further showed that esculetin had a concentration-dependent effect on cataractogenesis, and a strong correlation was observed between aldose reductase activity and lens opacification. Lastly, the administration of esculetin reduced the amount of aldose reductase in the lens.

The enzymatic distribution of aldose reductase activity was demonstrated by its localized immunoreactivity. Aldose reductase is primarily localized to epithelial cells and superficial cortical fibers of the lens (Lizak *et al.*, 1998). In the lenses of the GAL rats, increased immunoreactive positive staining of aldose reductase protein was observed in the epithelium, extending from the superficial region to the deeper cortex. However, esculetin inhibited the expression of aldose reductase protein in the lens epithelium and prevented its extension below the lens epithelium. Therefore, the decreased activity of aldose reductase observed in the esculetin-treated rats was likely due to reduced expression of aldose reductase protein.

In conclusion, our results show that the beneficial protective effect of esculetin against sugar cataract development occurs through the inhibition of aldose reductase. Moreover, a degree of protection against cataracts can likely be obtained through dietary intake of esculetin. Our results indicate that esculetin inhibits galactose-induced cataractogenesis by suppressing aldose reductase expression. Multiple mechanisms, such as non-enzymatic glycation and oxidative stress, are also involved in cataractogenesis as a consequence of hypergalactosemia or hyperglycemia. Therefore, further research is needed to investigate other possible mechanisms of action for esculetin.

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